45. A pharmaceutical composition comprising:

a) a recombinant nucleic acid molecule comprising a nucleotide sequence encoding a hepatitis C virus nonstructural protein, wherein said nucleotide sequence is operably linked to regulatory elements functional in human cells;

b) a pharmaceutically acceptable carrier or diluent; and

c) a facilitator;

wherein said regulatory elements functional in human cells comprise a promoter, enhancer, polyadenylation sequence, and a 5' untranslated region (5'-UTR), said 5'-UTR comprising at least the 9 most 3' nucleotides of a 5' UTR of hepatitis C virus.

REMARKS

Status of the Claims

Claims 3, 4, 6-8, 11-28 and 32-46 will be pending after entry of this amendment. Claims 6, 13, 32 and 45 have been amended. Support for the amendments can be found throughout the specification. For example, support can be found at page 10, l. 18 to page 11, l. 2. Claim amendments are for purposes of improved clarity or consistency of claim language unless otherwise noted. No claim amendment should be construed as an acquiescence in any ground of rejection. No new matter has been added by this amendment.

The rejection of claims 3, 4, 6-8, 11-28, and 32-46 under 35 U.S.C. § 101 has been withdrawn in the view of the applicants' arguments. The rejection of claims 3, 4, 6-8, 10-16, 45, and 46 under 35 U.S.C. § 102(e) has been withdrawn. Claims 3, 4, 6-8, 11-28 and 32-46 are rejected under 35 U.S.C. § 112, first paragraph, as containing non-enabled subject matter.

35 U.S.C. § 112 first paragraph

Claims 3, 4, 6-8, 11-28 and 32-46 were rejected under 35 U.S.C. § 112, first paragraph, for allegedly containing non-enabled subject matter. Applicants traverse this rejection.

The enablement requirement of § 112 is satisfied so long as a disclosure contains sufficient information that persons of ordinary skill in the art having the disclosure before them would be able to make and use the invention. *In re Wands,* 8 U.S.P.Q.2d 1400 (Fed. Cir.1988) (the legal standard for enablement under § 112 is whether one skilled in the art would be able to practice the invention without undue experimentation).

One skilled in the art would be able to practice the claimed invention without being required to perform undue experimentation. Applicants enclose a Declaration of Dr. Jack R. Wands under 37 C.F.R. § 1.132. In his Declaration, Dr. Wands states that the specification enables one skilled in the art to construct the claimed recombinant nucleic acid molecule, and the claimed pharmaceutical composition, method of inducing an immune response against hepatitis C virus in a human and method of treating a human infected with hepatitis C virus using the recombinant nucleic acid molecule.

> Utilizing information provided in the subject application, one skilled in the art can construct a recombinant nucleic acid molecule encoding hepatitis C virus NS4 or NS5 protein, ... operably linked to a promoter, enhancer, and polyadenylation sequence, ... and further operably linked to a 5'-untranslated region of hepatitis C virus as taught in the specification, ...
>
> Utilizing information provided in the subject application, as detailed in paragraph 5 [of the Declaration] and, for example, in Example 1 of the specification, "Design of HCV expression vectors," and using molecular biological techniques known in the art at the time of filing the application, one skilled in the art would be able to construct a recombinant nucleic acid molecule, [as claimed.]

See Wands Declaration, ¶ 5-7. Applicants provide an enabling disclosure that teaches one skilled in the art how to practice the claimed invention. Applicants' enabling disclosure

teaches the sequence of the entire HCV 5' UTR. See specification, for example, page 10, l. 26 to page 11, l. 2. The specification further enables a recombinant nucleic acid molecule comprising, in part, a 5' untranslated region comprising at least the 9 most 3' nucleotides of a 5' UTR of hepatitis C virus. The 5' untranslated region may further comprise at least the 50 most 3' nucleotides of a 5' UTR of hepatitis C virus, at least the 100 most 3' nucleotides of a 5' UTR of hepatitis C virus, at least the 150 most 3' nucleotides of a 5' UTR of hepatitis C virus, at least the 200 most 3' nucleotides of a 5' UTR of hepatitis C virus, at least the 250 most 3' nucleotides of a 5' UTR of hepatitis C virus, or at least the 300 most 3' nucleotides of a 5' UTR of hepatitis C virus. See specification, for example, page 10, l. 18-26.

One skilled in the art would understand the function of the 5' UTR of hepatitis C virus to include both positive and negative translational control elements within the 5'-UTR. One skilled in the art would be able to operably link the 5'UTR of hepatitis C virus to a recombinant nucleic acid molecule acting as an expression plasmid for proteins, for example, hepatitis C virus non-structural (NS) protein. See, for example, Yoo et al. *Virology* **191**: 889-899, 1992, (Exhibit A) and Wands Declaration, ¶ 6.

The specification teaches the design and construction of DNA expression vectors comprising HCV non-structural (NS) genes. DNA constructs expressing HCV genes NS3, NS4 and NS5 were PCR-cloned after inserting engineered start- and stop-codons as well as restriction enzyme sites utilizing specific PCR primers and restriction enzyme sites useful to construct the necessary expression vectors. See specification, for example, page 15, l. 22 to page 16, l. 17. One skilled in the art at the time the claimed invention was made would be able to use the nucleotide sequence of the 5' UTR of hepatitis C virus in combination with the PCR primers and restriction enzyme sites useful to construct the start and stop codons and the HCV NS3, NS4, and NS5 genes to obtain Applicants' claimed invention. As stated by Dr. Wands (Declaration ¶ 11), one skilled in the art utilizing information provided in the subject application would be able to construct the claimed invention, that is a recombinant nucleic acid molecule comprising a nucleotide sequence encoding hepatitis C virus non-structural

(NS) proteins wherein said nucleotide sequence is operably linked to regulatory elements, said regulatory elements comprising a promoter, enhancer, polyadenylation sequence, and a 5' untranslated region (5'-UTR) of hepatitis C virus.

The Examiner points to a statement by Selby et al. that "better expression was observed with constructs containing deletions in the 5' UTR than with full-length 5' UTR." This statement in Selby et al., (see, for example, p. 1105, Col. 2) does not lead one to the conclusion stated by the Examiner that "it is not clear whether the claimed construct would produce sufficient protein to produce an immune response." See Office Action, Paper No. 11, page 3. The absolute quantity of protein produced by a DNA expression vector does not necessarily correlate with the level of immune response in a mammalian subject. In particular, the results of Tokushige et al., *Hepatology*, **24**:14-20, 1996 (Exhibit B) indicate that the intact HCV 5' UTR provides sufficient intracellular expression of HCV core protein to produce immune reactive HCV core protein on a Western blot. See Tokushige et al., Figure 1B. Because protein expression from a construct comprising the HCV 5' UTR would produce lower levels of gene expression, it does not necessarily follow, as urged by the Examiner, that "it is not clear whether the claimed construct would produce sufficient protein to produce an immune response." As discussed in further detail below, one skilled in the art would know that the claimed construct used to immunize a human subject would produce an immune response.

In his Declaration, Dr. Wands states that contrary to the disclosure of Tokushige et al., the specification enables one skilled in the art to use the claimed composition and the claimed method of inducing an immune response to stimulate a strong humoral immune response and a strong cytotoxic T-cell (CTL) response.

> "... the results shown in Examples 1 through 7 of the subject application taken with results shown in Tokushige et al., enable one skilled in the art to conclude that immunization of a human with a pharmaceutical composition comprising a recombinant nucleic acid molecule comprising a nucleotide sequence encoding hepatitis C virus NS4 or NS5 protein wherein said nucleotide

> sequence is operably linked to a promoter, enhancer, polyadenylation sequence, and 5' untranslated region (5'-UTR) of hepatitis C virus would stimulate a strong humoral immune response and a strong specific $CD8^+$ cytotoxic T-cell (CTL) response.
>
> "In support of these conclusion, non-structural (NS) proteins are better than core proteins as antigens to stimulate a cell mediated and a humoral immune response. Strong specific $CD8^+$ cytotoxic T-cell (CTL) response and *in vivo* CTL activity in a mouse tumor model is generated for non-structural (NS) proteins, for example, NS3 and NS5."

See Wands Declaration, ¶ 8-10. Dr. Wands states that the claimed composition and the claimed method of inducing an immune response will stimulate a strong humoral immune response and a strong cytotoxic T-cell (CTL) response. This statement is supported by statements in the specification and in Encke et al., *J. Immunol.*, **161**:4917-4923, 1998 (Exhibit C) that a pharmaceutical composition or a method of inducing an immune response with a recombinant nucleic acid molecule expression HCV non-structural (NS) proteins stimulate a superior humoral and CTL response than recombinant nucleic acid molecules expressing the HCV core protein.

With respect to the teachings of a specification disclosure, the following statement from *In re Manocchi*, 169 U.S.P.Q. 367, 369-370 (C.C.P.A. 1971), is noteworthy:

> The only relevant concern of the Patent Office under these circumstances should be over the truth of any such assertion. The first paragraph of § 112 requires nothing more than objective enablement. How such a teaching is set forth, either by the use of illustrative examples or by broad terminology, is of no importance.
>
> As a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirements of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied upon for enabling support.
> *In re Manocchi*, 169 U.S.P.Q. 367, 369-370 (C.C.P.A. 1971).

The examiner has no reason to doubt the objective truth of the statements contained in the application teaching the manner and process of making the claimed invention. Applicants' claimed invention is fully enabled as a nucleic acid molecule, a pharmaceutical composition and a method of inducing an immune response with a recombinant nucleic acid molecule comprising genes for NS proteins and the 5'-UTR of hepatitis C virus. The specification discloses and one skilled in the art would know, for example, as evidenced by Dr. Wands' declaration, and by Tokushige et al. and Encke et al., that the NS proteins expressed by the claimed nucleic acid molecule would produce a sufficient humoral and CTL immune response in a human. The examiner has not provided evidence sufficient to doubt the truth of the disclosure stating that the claimed compositions and methods produce a sufficient immune response in a human.

Immunizing mice with a DNA vaccine against HCV and measuring an immune response is an accepted model for DNA vaccination against HCV to raise a therapeutic immune response in a human subject. See Wands Declaration, ¶ 12-13. The examples in the instant specification vaccinated and immunized mice with a DNA vaccine but did not challenge the immunized mice with HCV. Rather, these examples measured efficacy of genetic immunization with DNA vectors expressing HCV NS5 protein as a function of *in vivo* tumor growth and *in vitro* splenocyte activation. The experimental evidence presented in the specification demonstrated that immunization and assessment of cytotoxic T-lymphocytes (CTL assay) in mice is a relevant measure of cell mediated immunity leading to immunity against HCV infection. The experimental evidence in the examples in the specification correlates an *in vitro* or *in vivo* animal model assay to a disclosed or claimed pharmaceutical composition or a method of inducing an immune response against hepatitis C virus in a human.

> [I]f the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the

> examiner has evidence that the model does not correlate. Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition.
> *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995)
>
> Since the initial burden is on the examiner to give reasons for the lack of enablement, the examiner must also give reasons for a conclusion of lack of correlation for an *in vitro* or *in vivo* animal model example. A rigorous or an invariable exact correlation is not required, as stated in *Cross v. Iizuk*a, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985).
> See MPEP 2164.02.

The Examiner provides no evidence that a correlation does not exist between a cytotoxic T cell (CTL) assay, as described in the instant examples, and efficacy of a genetic vaccine to produce a therapeutic immune response against HCV. A specific CTL assay accepted in the art is shown, for example, in Example 6 of the specification. See specification, for example, page 21, l.1 to page 22, l. 8. The assay in Example 6 demonstrates specific CTL activity of spleen cells derived from mice immunized with pApNS5 construct compared to mice immunized with a mock DNA construct (no HCV DNA). The specific *in vitro* CTL activity is from spleen cells of mice that have been challenged with myeloma cells (either mock or HCV transfected), in which the spleen cells have not been prestimulated with HCV or HCV peptides. Figure 3 of the specification demonstrates that spleen cells from pApNS5 immunized mice show a specific CTL activity greater than the spleen cells of mock DNA immunized mice. This is a standard and accurate measure of CTL activity in response to immunization with a genetic vaccine or for immunization with a standard subunit vaccine. Furthermore a specific assessment of cytotoxic T-lymphocyte activity *in vivo* is demonstrated by reduction of tumor weight and volume in mice immunized with the pApNS5 construct compared to mice immunized with a mock DNA construct (no HCV DNA). See, for example, Example 7, page 22, l. 9 to page 23, l. 17. Both the CTL assay and the reduction in tumor volume are *in vivo* assays accepted in the art. These assays are predictive of efficacy of a vaccine to treat HCV infection.

The evidence of record supports the conclusion that one skilled in the art at the time the invention was made would be able to make and use the claimed invention without undue experimentation. One skilled in the art would readily be able to make Applicants' claimed nucleic acid molecules, prepare pharmaceutical compositions using these nucleic acid molecules, and use such compositions to immunize an individual without undue experimentation. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 112, first paragraph regarding enablement be withdrawn.

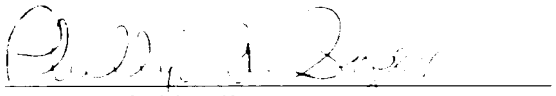
CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 206-332-1380.

Respectfully submitted,

Date: January 24, 2003



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VERSION WITH MARKINGS TO SHOW CHANGES MADE

6. (Amended four times) A recombinant nucleic acid molecule comprising a nucleotide sequence encoding hepatitis C virus NS4 or NS5 protein wherein said nucleotide sequence is operably linked to regulatory elements, said regulatory elements comprising a promoter, enhancer, polyadenylation sequence, and a 5' untranslated region (5'-UTR), said 5'-UTR comprising at least the 9 most 3' nucleotides of a 5' UTR of hepatitis C virus.

13. (Amended four times) A pharmaceutical composition comprising:

a) a recombinant nucleic acid molecule comprising a nucleotide sequence encoding hepatitis C virus NS4 or NS5 protein, wherein said nucleotide sequence is operably linked to regulatory elements functional in human cells; and

b) a pharmaceutically acceptable carrier or diluent;

wherein said regulatory elements functional in human cells comprise a promoter, enhancer, polyadenylation sequence, and a 5' untranslated region (5'-UTR), said 5'-UTR comprising at least the 9 most 3' nucleotides of a 5' UTR of hepatitis C virus.

32. (Amended four times) A method of treating a human who is infected with hepatitis C virus comprising administering to said human a pharmaceutical composition in an amount effective to induce a therapeutic immune response against hepatitis C virus, wherein said composition comprises a recombinant nucleic acid molecule comprising a nucleotide sequence encoding a hepatitis C virus nonstructural protein, wherein said nucleotide sequence is operably linked to regulatory elements functional in human cells, and a pharmaceutically acceptable carrier or diluent, wherein said regulatory elements functional in human cells comprise a promoter, enhancer, polyadenylation sequence, and a 5' untranslated region (5'-UTR), said 5'-UTR comprising at least the 9 most 3' nucleotides of a 5' UTR of hepatitis C virus.

45. (Twice Amended) A pharmaceutical composition comprising:

a) a recombinant nucleic acid molecule comprising a nucleotide sequence encoding a hepatitis C virus nonstructural protein, wherein said nucleotide sequence is operably linked to regulatory elements functional in human cells;

b) a pharmaceutically acceptable carrier or diluent; and

c) a facilitator;

wherein said regulatory elements functional in human cells comprise a promoter, enhancer, polyadenylation sequence, and a 5' untranslated region (5'-UTR), said 5'-UTR comprising at least the 9 most 3' nucleotides of a 5' UTR of hepatitis C virus.

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EXHIBIT A

VIROLOGY 191, 899–909 (1992)

NOTICE: THIS MATERIAL MAY BE PROTECTED BY COPYRIGHT LAW (TITLE 17, U.S. CODE)

5′ End-Dependent Translation Initiation of Hepatitis C Viral RNA and the Presence of Putative Positive and Negative Translational Control Elements within the 5′ Untranslated Region

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Hepatitis C virus (HCV) is a distant relative of pestiviruses and flaviviruses, but it has a 5′ untranslated region (UTR) with some features structurally similar to that of picornaviruses. In order to test the role of the 5′ UTR in controlling the expression of the HCV polyprotein, we fused full-length or deleted versions of the 5′ UTR of HCV-1 RNA to chloramphenicol acetyl transferase (CAT) mRNA to monitor CAT activity *in vivo*. We found: (1) the full-length 5′ UTR of HCV-1 RNA is translationally inactive while 5′ deletions which mimic a 5′ subgenomic RNA detected *in vivo* are active, (2) an efficient *cis*-acting element which represses translation is found at the 5′ terminus, (3) a putative element which enhances translation is found near the 3′ terminus of the 5′ UTR, (4) additional *cis*-acting elements including small open reading frames (ORFs) upstream from the putative enhancer element downregulate translation. We did not find evidence supporting the existence of an internal ribosome entry site in the 5′ UTR of HCV-1 RNA. These data suggest that HCV may employ a distinctive translation control strategy such as the generation of subgenomic viral mRNA in infected cells. Translational control of HCV might be responsible for some of the characteristic pathobiology seen in viral infection. © 1992 Academic Press, Inc.

INTRODUCTION

HCV is the major etiologic agent of non-A, non-B hepatitis worldwide (Alter *et al.*, 1989; Choo *et al.*, 1989, 1990; Kuo *et al.*, 1989). It has a positive-strand RNA genome of approximately 9500 nucleotides which encodes a polyprotein that is processed into structural and nonstructural proteins (Choo *et al.*, 1989, 1990, 1991). HCV resembles flaviviruses and pestiviruses in genome organization within the polyprotein region and is proposed to be a member of the *Flaviviridae* (Choo *et al.*, 1990, 1991; Han *et al.*, 1991; Houghton *et al.*, 1991). HCV isolates show considerable amino acid sequence variations in coding regions, but they can be segregated into at least four different groups based on distinct amino acid sequence patterns (Houghton *et al.*, 1991; Okamoto *et al.*, 1992).

The 5′ untranslated region (UTR) of full-length HCV RNA appears to be 341 nucleotides long, based on at least five putative full-length HCV clones reported to date (Chen *et al.*, 1992; Han *et al.*, 1991; Tanaka *et al.*, 1992; Okamoto *et al.*, 1991). Unlike the polyprotein region, the 5′ UTR of HCV isolates are highly conserved (>98% within a group or >93% between groups), suggesting a functional importance (Han *et al.*, 1992). This region contains up to five upstream open reading frames (ORFs), the first four of which are overlapping in HCV-1, the prototype HCV isolate (Choo *et al.*, 1991; Han *et al.*, 1991). The 5′ UTR is homologous in nucleotide sequence to pestiviruses, especially in four regions (PEST-I to -IV) (Fig. 1C) (Han *et al.*, 1991). Previously, primer extension analysis has revealed that two prominent species of HCV RNA exist in samples derived from infected patients (Han *et al.*, 1991): a longer presumptive full-length genomic RNA, the 5′ terminus of which is predicted to form a hairpin structure (Chen *et al.*, 1992; Han *et al.*, 1991; Inchauspe *et al.*, 1991; Okamoto *et al.*, 1991, 1992), and a shorter 5′ subgenomic RNA, the 5′ terminus of which starts 145 nucleotide from the 5′ terminus of the longer RNA (Han *et al.*, 1991). These features suggest that control element(s) important to viral replication and polyprotein translation may be present in this region of the HCV genome.

The 5′ end of the HCV genome is considerably different from flaviviruses (Han *et al.*, 1991). However, it has been suggested that the presence of small upstream ORFs and the relatively long length of the HCV leader (Choo *et al.*, 1991; Han *et al.*, 1991; Inchauspe *et al.*,

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1991; Kato *et al.*, 1990; Takamizawa *et al.*, 1991) are features reminiscent of poliovirus 5′ UTRs (Kitamura *et al.*, 1981). The 5′ UTRs of picornaviruses allow for cap-independent translation of picornaviral genomes and also contain a *cis*-acting site which allows for internal entry of ribosomes (Jang *et al.*, 1989; Pelletier and Sonenberg, 1988) in contrast to the more usual scanning mechanism hypothesized to account for the translation of most eukaryotic capped messages (Kozak, 1983, 1989). In fact, Tsukiyama-Kohara *et al.* (1992) recently reported the presence of an internal ribosome entry site (IRES) within the 5′ UTR of HCV RNA prepared from two Japanese isolates using an *in vitro* system. However, we report evidence using an *in vivo* system employing both monocistronic and dicistronic contructs, which indicates that the translation of HCV-1 RNA is mediated not by an internal initiation but by a 5′ end-dependent initiation which favors the conventional cap-dependent ribosome scanning mechanism. Moreover, we have mapped three distinct *cis*-acting control elements, which can block, repress, or enhance translation. Our results suggest that a subgenomic mRNA which is generated by an unknown mechanism *in vivo* may be the major template for the translation of the viral polyprotein.

MATERIALS AND METHODS

Cells, bacterial strains, and plasmids

Huh7, HeLa, and HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (GIBCO-BRL, Gaithersburg, MD). Cells were grown in the presence of 7% CO_2. All plasmids were grown in *Escherichia coli* HB101, purchased from GIBCO-BRL.

Enzymes

Restriction enzymes and T4 DNA ligase were purchased from Boehringer-Mannheim (Indianapolis, IN), Taq-polymerase from Perkin Elmer (Norwalk, CT), and T7 RNA polymerase and RNasin from Promega (Madison, WI).

Construction of expression plasmids

The construction of plasmid pT7EMCAT and pSV_2CAT have been described (Elroy-Stein *et al.*, 1989; Gorman *et al.*, 1982). Plasmid pHCVCAT was constructed by attaching *Hin*dIII sites at the both ends of the 5′ UTR of HCV cDNA (Han *et al.*, 1991) by PCR (Saiki *et al.*, 1988) and cloning the resultant fragment into the *Hin*dIII site of pSV_2CAT. Plasmid pEQ355 was constructed by inserting the 341 bp 5′ UTR of HCV-1 into the *Hin*dIII/*Asp*718 sites resident in the multiple cloning site of β-galactosidase (β-gal) expression plasmid, pEQ176 (Schleiss *et al.*, 1991). The HCV-1 5′ UTR was generated as a *Hin*dIII/*Asp*718 PCR fragment using 3114, an *Eco*RI fragment from a lambda vector (Houghton and Lee, unpublished data), as template. Plasmid pEQ391 [pCMV(CAT/HCV/LacZ)], was generated by ligating a 716-bp *Hin*dIII/*Ban*I fragment encoding the CAT gene isolated from plasmid pSV_2CAT (Gorman *et al.*, 1982) into plasmid pEQ355 at the *Hin*dIII site. The *Hin*dIII sites were ligated and the *Ban*I site and unligated *Hin*dIII site in pEQ355 were blunted with Klenow and religated. Plasmid pEQ416 [pCMV(CAT/polio/LacZ)] was constructed by ligating a 716-bp *Hin*dIII/*Bam*HI CAT-gene-encoding PCR fragment generated using pSV_2CAT as template, a 995-bp *Bam*HI/*Xho*I fragment encoding the poliovirus 5′ UTR isolated from pEQ396 (Spaete *et al.*, unpublished data), along with β-gal expression plasmid pEQ176 digested at the *Hin*dIII/*Xho*I site in the polylinker. pEQ396 is a β-gal expression plasmid constructed by cloning the 5′ UTR poliovirus sequence taken from pLNPOZ (Adam *et al.*, 1991) as an *Xho*I/*Pst*I fragment blunted using Klenow into pEQ377 digested at *Xba*I/*Sna*BI sites in the polylinker. The *Xba*I site was also filled with Klenow to create blunt ends. Transcription of β-gal in pEQ377 is promoted by T7 bacteriophage promoter (Geballe, unpublished data). Plasmid p(CAT/SV40/LacZ) was constructed by ligating the 716-bp *Hin*dIII/*Bam*HI CAT gene encoding PCR fragment described above, along with SV40 polyadenylation signals contained in an 847-bp *Bgl*II/*Bam*HI fragment isolated from pPR25 (Burke, unpublished data), and β-gal expression plasmid pEQ176 digested with *Hin*dIII/*Bgl*II. The authenticity of all PCR products was verified by sequencing each of the resulting segments in the plasmids (Chen and Seeburg, 1985).

Construction of hybrid CAT RNAs

Segments of pSV_2CAT vectors indicated by arrows (Fig. 1) were amplified by PCR as described (Saiki *et al.*, 1988; Shyamala and Ames, 1991). Each sense primer (PSV or P1 to P9) was designed to have a bacteriophage T7 promoter (TAATACGACTCACTATAG) at the 5′ end and a SV40 or HCV sequence of 16 to 18 bases at the 3′ end (Table 1). An antisense primer (PT30, Table 1) had a stretch of 40 Ts at the 5′ end and a complementary SV40 sequence (GGAGGAGTAG) at the 3′ end; this sequence binds to vectors 350 bp after a stop codon in the CAT gene by virtue of a perfect match of 10 nucleotides and an additional poly A track present in the template DNA. A segment of pT7EMCAT was amplified by primers T7 and T30 (Table 1). Each PCR product was transcribed by T7 polymerase with or

TABLE 1

LIST OF OLIGONUCLEOTIDES USED AS PRIMERS AND PROBE

Oligonucleotide	Size (nucleotide)	Position in HCV genome	Sequence (5′ to 3′)
P1	34	1 to 16	TAATACGACTCACTATAGGCCAGCCCCCTGATGG
P2	38	23 to 40	TAATACGACTCACTATAGCACTCCACCATGAATCAC
P3	36	35 to 52	TAATACGACTCACTATAGAACACTCCCCTGTGAGG
P4	36	83 to 100	TAATACGACTCACTATAGCCATGGCGTTAGTATGAG
P5	36	99 to 116	TAATACGACTCACTATAGAGTGTCGTGCAGCCTCCA
P6	37	145 to 162	TAATACGACTCACTATAGGGTCTGCGGAACCGGGTA
P7	36	218 to 235	TAATACGACTCACTATAGCCTGGAGATTTGGGCGTG
P8	36	255 to 272	TAATACGACTCACTATAGGAGTAGTGTTGGGTCGCG
P9	36	322 to 339	TAATACGACTCACTATAGGGTCTCGTAGACCGTGCA
PSV	36	—	TAATACGACTCACTATAGATTCCAGAAGTAGTGAGG
T7	18	—	TAATACGACTCACTATAG
pT30	50	—	T_{40}GGAGGAGTAG
T30	45	—	T_{30}CAGGCGTAGCACCAG
JHC271	30	—	GGGATATATCAACGGTGGTATATCCAGTGA

Note. The position of each oligonucleotide in the HCV genome is based on Han *et al.* (1991).

without cap analogue (Promega, p2010), treated with DNase, extracted with phenol–chloroform, and precipitated twice with ethanol in the presence of 2.5 *M* ammonium acetate. Concentration of each poly(A)+ RNA was estimated by uv absorption and confirmed by Northern and dot-blot hybridization (Fig. 2) as described (Han *et al.*, 1986) using JHC271 as a probe (Table 1). In SV*CAT, R11, R13, and R14, sequences were internally inserted or deleted by an overlapping PCR method (Shyamala and Ames, 1991). The PCR products were confirmed to be correct by sequencing.

Translation of hybrid CAT RNAs *in vitro*

Synthetic RNAs were translated in nuclease-treated rabbit reticulocyte lysate (GIBCO-BRL) in the presence of 140 m*M* potassium acetate, as suggested by the manufacturer. Additional studies examining the influence of K^+ ion concentration on cap dependence were done in the presence of 50, 100, 150, and 200 m*M* potassium acetate. Aliquots of the translation product labeled with [^{35}S]methionine were analyzed by electrophoresis in a 12% polyacrylamide gel as previously described (Laemmli, 1970).

Transfection of hybrid CAT RNAs into mammalian cells for CAT assay

Two micrograms of each synthetic RNA was transfected into 1 × 10^6 cells in a 3.5-cm Costar plate (Thomas Scientific, Swedesboro, NJ) using 15 μg of lipofectin (GIBCO-BRL) according to the procedure of Feigner *et al.* (1987) modified by the manufacturer. Cells were incubated overnight and harvested for CAT assay as previously described (Gorman *et al.*, 1982). The relative CAT activity was shown to be linear between 0.5 and 5 μg of transfected RNA. Post-transfection incubation between 6 hr and overnight did not significantly affect CAT activity. For translation of RNAs in poliovirus-infected cells, Huh7 cells were infected with poliovirus (Mahoney strain, ATCC VR-59) at a multiplicity of infection (m.o.i.) of 100. Cells were transfected with RNAs 2 hr after the infection and harvested 4 hr after the transfection. Cells maintained normal morphology during the 6-hr infection, after which they began to change shape and detach from the culture dish.

Transfection of dicistronic DNA constructs into cells

Twenty micrograms of each plasmid DNA purified by a banding in a CsCl gradient were transfected into 2 × 10^5 Huh7 cells by a calcium phosphate method (Gorman *et al.*, 1982). The cells were harvested 48 hr after transfection and cell extract was prepared by repeated freezing and thawing. The CAT assay was performed as described (Gorman *et al.*, 1982). The LacZ assay was according to Miller (1972).

RESULTS

Construction of RNAs with deletions in the 5′ UTR of the HCV genome and rationale for the method

In order to map *cis*-acting element(s) controlling translation in the HCV genome, we linked full-length (from nucleotide 1 to 341) or deleted versions of the 5′ UTR of HCV-1 RNA to the coding region of chloramphenicol acetyl transferase (CAT) mRNA (Figs. 1 and 2)

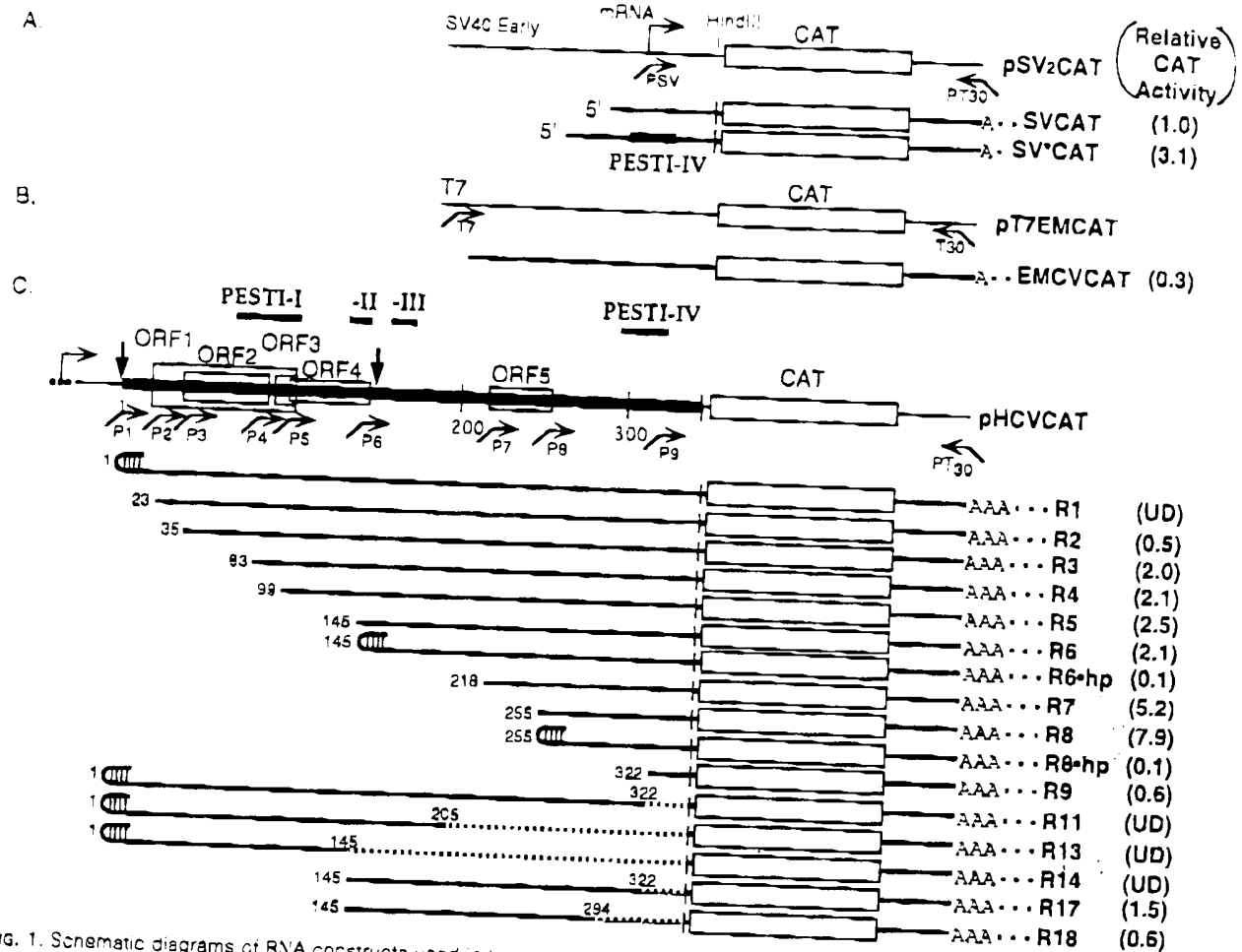

FIG. 1. Schematic diagrams of RNA constructs used in transient expression analysis. (A) SVCAT RNA. (B) EMCVCAT RNA. (C) Engineered HCVCAT RNAs (R1 to R18) with their relative CAT activities in Hun 7 cells. Position of open reading frames (ORF1 to ORF5), pestivirus homology boxes (PESTI-I to PESTI-IV), and the 5' end of the two prominent HCV RNAs (two vertical arrows) are marked in pHCVCAT. Numbers presented in each RNA indicates the nucleotide position in the wild type HCV sequence (Han *et al.*, 1991). CAT activity is given as an average of three independent measurements. Horizontal arrows indicate primers used to amplify transcription templates. UD refers to undetectable. Dashed lines indicate 3' deletions.

and measured CAT protein expression *in vitro* (data not shown) and CAT enzymatic activity *in vivo* (Fig. 3). We synthesized each RNA by transcribing a DNA fragment with T7 polymerase, which was first amplified by PCR to contain a specific 5' or 3' deletion (Fig. 1). Each RNA was designed to have a cap at the 5' end and poly A tail (A40) at the 3' end to increase stability in cells. This approach allows an efficient production of a large amount of RNA with uniformly defined 5' and 3' ends (Fig. 2). Unlike conventional DNA transfection strategies, RNA transfection of cells using this approach circumvents possible splicing and transport problems that certain RNA molecules may encounter in the nucleus. By the same method, we synthesized two additional RNAs: (1) the SVCAT with the 5' leader of SV40 early mRNA that served as a positive control for a conventional cap-dependent translation (Kozak, 1989) and (2) the EMCVCAT with the 5' leader of EMCV that served as a positive control for cap-independent internal initiation (Jang *et al.*, 1989).

Translation of hybrid CAT RNAs *in vitro*

In order to test whether synthetic RNAs were biologically active and to determine their translational profile *in vitro*, we translated these RNAs in rabbit reticulocyte lysate (data not shown). All RNAs including SVCAT generated a CAT protein of the expected size. The *in vitro* results can be summarized as follows: (1) in HCVCAT constructs, R1 to R5 produced CAT protein, but only at barely detectable levels. This level of translation gradually increased in R6 and in R7, reaching a

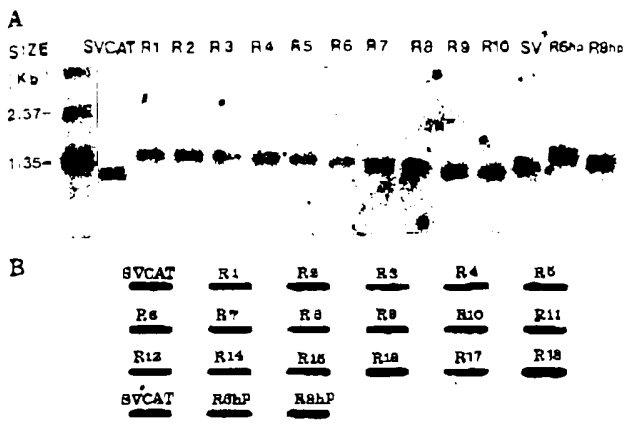

FIG. 2. Northern blot (A) and slot blot analysis (B) of engineered hybrid CAT RNAs. Two-tenths of a microgram of each denatured RNA was electrophoresed in a 1.5% agarose gel containing 0.3 *M* formamide for Northern blot analysis or directly blotted on the filter for slot blot analysis (Han *et al.*, 1985). Each blot was hybridized with a ^{32}P-labeled cHC271 which binds to the 5′ terminus of the CAT coding region.

maximum 12-fold increase in R8. (2) At K^+ concentrations of 140 m*M* the *in vitro* translation of capped SVCAT and HCVCAT RNAs (R7, R8) was more efficient than that of the uncapped RNAs by an average of 20-fold. (3) At lower K^+ concentrations (50 to 100 m*M*), translation of uncapped R1 template generated CAT protein at levels comparable to that of the capped R1 template, possibly indicating the occurrence of weak internal initiation. These results, however, did not confirm recent data by Tsukiyama-Kohara *et al.* (1992) who reported the detection of an efficient internal ribosome entry site within the 5′ UTR of HCV RNA using rabbit reticulocyte lysate and HeLa cell extracts. Because of this discrepancy and the fact that protein synthesis *in vitro* using cell lysates does not always faithfully represent translation conditions *in vivo* (Kozak, 1983), we elected to test our constructs in an *in vivo* system by transfecting mono- and dicistronic templates directly into mammalian cells as a more relevant readout of biological activity.

Translation of hybrid CAT RNAs *in vivo* and identification of control elements

In order to determine the translation profile of the monocistronic constructs *in vivo*, we transfected RNAs (R1 to R18) along with the control RNA, SVCAT, into a human hepatocyte cell line (Huh7) using lipofectin (Felgner *et al.*, 1987) and monitored CAT activities (Fig. 3). In the full-length construct R1, CAT activity was repeatedly undetectable (Fig. 3, lane 3) unless the amount of RNA was increased by 5-fold and more cell extract was used (data not shown). This result suggested that the full-length HCV RNA may not be an efficient translation template *in vivo*. When a series of 5′ deletion constructs were analyzed (Fig. 3, lanes 4–11), CAT activity was first detected in R2 in which the 5′ terminal hairpin of 23 nucleotides was removed. This activity increased by 4-fold in R3 and a similar level of activity was detected in R4, R5, and R6 which were systematically deleted for ORF1 to 4. It should be noted that the 5′ leader sequence in R6 was identical to that of the 5′ subgenomic RNA detected *in vivo* (Han *et al.*, 1991). This activity further increased by 2-fold in R7 in which the AUG codon of ORF 5 was removed and an additional 1.5-fold in R8 which retains only 86 nucleotide of 3′ proximal sequence, representing a maximum activity. These data suggested that sequences upstream from nucleotide 255 including the small ORFs are inhibitory to the translation from the major initiation codon for the polyprotein.

The maximum CAT activity seen in R8 decreased sharply upon a further deletion of 67 nucleotides (Fig. 3, R9). This result suggested that an efficient positive control element that stimulates translation may be present downstream from nucleotide 255. This 86-nucleotide region contains a 28-nucleotide sequence at position 291 to 281 with 90% sequence identity to pestiviruses and has been designated as PEST-IV (Han *et al.*, 1991). To determine whether the PEST-IV element is solely responsible for the observed translation stimulation, we performed 3′ deletion analysis on R6 (Fig. 3, lanes 12–15). We chose this RNA because any construct which contained an intact 5′ terminus of HCV RNA was inactive (see below) and 3′ deletion in R8 would generate RNA with a short 5′ leader. Upon transfection, the CAT activity seen in R6 was dropped 1.5-fold by a deletion of 20 nucleotides from the 3′ end of R6 (R17, lane 14) and a further 2.5-fold decrease by an additional deletion of 28 nucleotides (R18, lane 15). These data indicated that additional upstream and downstream sequences from the PEST-IV were neces-

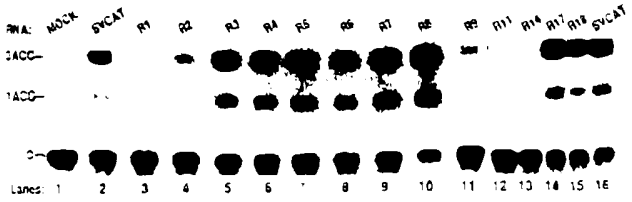

FIG. 3. Translation of transfected synthetic RNAs in Huh7 cells. Two micrograms of each RNA were transfected into 1×10^6 Huh7 cells using lipofectin (Felgner *et al.*, 1987) and the CAT activity was measured 14 hours later (Gorman *et al.*, 1982). The positions of unacetylated CAT (C) and monoacetylated CAT products (1ACC or 3ACC) are indicated.

sary for maximum translational enhancement. Nonetheless, this PEST-IV sequence appears to be a part of a positive *cis*-acting element which can be transferred to a heterologous 5′ leader: when this 28-nucleotide sequence was inserted into the SVCAT to create SV*CAT, it conferred an increase in CAT activity of 3-fold (Fig. 4, lane 2 vs lane 3).

In additional 3′ deletion analysis, no CAT activity was detected in R11 and R14 (Fig. 3, lanes 12, 13) or in R13 (data not shown), all of which contained the 5′ hairpin. These data are consistent with the view that the 5′ hairpin may be inhibitory to the translation of HCV RNA.

The effect of 5′ hairpin of HCV on the translation of CAT RNAs

Since RNAs with the intact 5′ terminus were all inactive irrespective of the downstream sequences (Fig. 3, lanes 3, 12, 13), we tested whether a potential 5′ hairpin structure resident in the most distal 23 nucleotides (hereafter referred to as the 5′ hairpin) is directly responsible for the observed translation inhibition. Accordingly, the 5′ hairpin was linked to the 5′ terminus of the two active RNAs, R6 and R8. These RNAs (R6hp, R8hp) were transfected into Huh7 cells and the CAT activity was measured. As shown in Fig. 4, the juxtaposition of the hairpin on these constructs nearly abolished the translation as demonstrated by the relative CAT activity (lane 7 vs lane 9, lane 10 vs lane 12). This indicates that the 5′ hairpin is a potent translation inhibitor, although complete inhibition requires more sequence than the 23-nucleotide hairpin alone.

Translation of hybrid CAT RNAs in poliovirus-infected cells

Poliovirus infection is known to inhibit the cap-dependent translation of cellular mRNA and thereby promote translation of its own or heterologous RNA which contains an IRES within its 5′ UTR (Jang *et al.*, 1989; Macejak and Sarnow, 1991; Pelletier and Sonenberg, 1988). This inhibition is believed to be mediated indirectly by the poliovirus-encoded proteinase 2A by activating an unidentified latent cellular protease which in turn cleaves p220, a component of the cellular cap-binding protein complex (eIF-4F) (Sonenberg, 1988). Therefore, we transfected hybrid CAT RNAs with various 5′ UTR into Huh7 cells infected with poliovirus. This strategy was designed to determine the cap dependency of each RNA and to detect the possible existence of a weak IRES which may be present in the HCV 5′ UTR of HCV-1 RNA. As expected, poliovirus infection increased CAT activity by sevenfold in EMCVCAT (Fig. 4, lane 13 vs lane 14), a positive control RNA for internal initiation (Elroy-Stein *et al.*, 1989). In contrast, the

FIG. 4. The effect of poliovirus infection, the 5′ hairpin, and the PEST-IV element on CAT activity in Huh7 cells. Cells (1×10^6) were uninfected or infected (lane 4, 8, 11, and 14) with poliovirus type 1 (ATCC VR-59) 2 hr before RNA transfection at a m.o.i. of 100. Transfection conditions were the same as in Fig. 3 except that CAT activity was assayed 4 hr after RNA transfection.

infection substantially decreased CAT activity in SVCAT (lane 2 vs lane 4) as well as in two HCV constructs, R6 (lane 7 vs lane 8) and R8 (lane 10 vs lane 11), respectively. The lowered CAT activities seen in poliovirus-infected cells were further diminished if infected cells were incubated longer than 2.5 hr prior to RNA transfection (data not shown). The CAT activity of R1 remained undetectable regardless of poliovirus infection (lanes 5, 6). This result strongly suggested that an IRES is not present in the 5′ UTR of HCV-1 RNA.

With the exception of the R1 construct, the constructs tested in the above experiment contained large deletions of the 5′ UTR. Because it is formally possible that such deletions may have affected a putative IRES structure and/or function, we elected to test constructs with less extensive deletions for their ability to translate CAT protein in poliovirus-infected cells. In agreement with results shown in Fig. 4, the CAT activity of the R1 construct remained undetectable in the presence or absence of poliovirus infection (Fig. 5A, lanes 3 and 4). Constructs R2 to R5 showed relative levels of CAT activity similar to those described previously (e.g., Fig. 3) when tested in the absence of poliovirus infection (Fig. 4A, lanes 5, 7, 9, and 11, respectively). However, the CAT activities of the HCV leader templates were practically abolished in the poliovirus-infected cells (Fig. 5A, lanes 6, 8, 10, and 12).

In addition, templates SVCAT and R1 to R3 were tested in a similar protocol using uncapped messages. As is shown in Fig. 5B, the uncapped templates were inactive in transfected cells whether or not the cells were subsequently infected with poliovirus. These results strongly suggest that monocistronic messages with *cis*-acting regulatory elements derived from the HCV 5′ UTR are translated by a cap-dependent mecha-

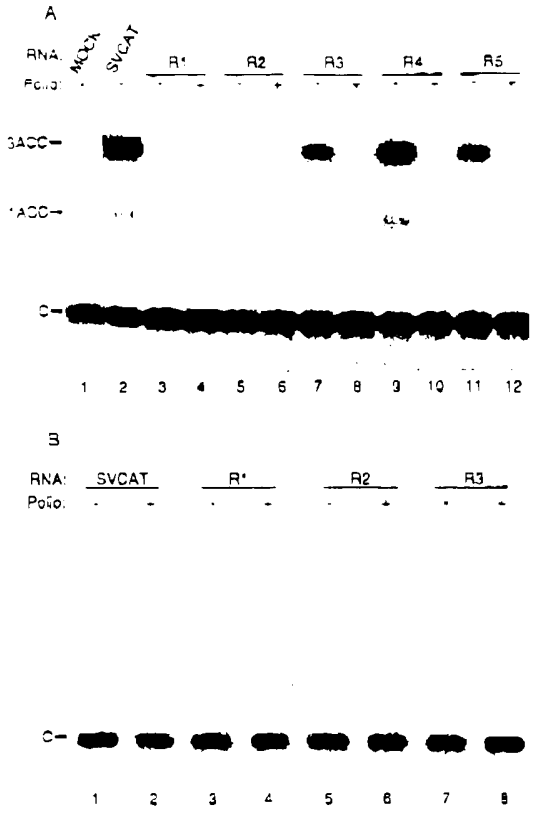

FIG. 5. The effect of poliovirus infection on CAT activity of capped (A) or uncapped (B) RNAs in Huh7 cells. Cells (1×10^6) were uninfected or infected (lanes 4, 6, 8, 10, and 12 for A; lanes 2, 4, 6, and 8 for B) with poliovirus, transfected with indicated RNAs and assayed for CAT activity. Experimental conditions were the same as in Fig. 4.

nism and that the HCV 5'-noncoding region does not have an IRES element.

Translation of dicistronic mRNA in Huh7 cells

The possible presence of an IRES within the 5' leader of HCV was further tested by transfecting Huh7 cells with DNA constructs designed to transcribe a dicistronic mRNA. Thus we placed the 5' UTR of HCV RNA as an intercistronic spacer between CAT as the first cistron and LacZ as the second cistron and cloned this linked DNA into an expression vector, in which transcription is derived by the strong enhancer-promoter of the major immediate-early gene in cytomegalovirus (CMV) (Fig. 6A). In addition, we constructed both positive and negative control dicistronic vectors, in which the 5' UTR of HCV was replaced with the 5' UTR of poliovirus and the 3' UTR of SV40 early gene, respectively. Upon transfection into Huh7 cells, all three constructs supported translation of the first CAT cistron at a comparable level (Fig. 6B); however, the dicistronic construct with the HCV leader did not support the translation of the second LacZ cistron at a level comparable to the dicistronic control construct employing a poliovirus leader (Fig. 6C). These data support the earlier evidence generated using monocistronic constructs that the full-length 5' UTR of HCV genome does not contain an IRES.

Translation of HCV RNA constructs in HeLa and HepG2 cells

Transient transfection assays can give different readouts that are cell line dependent. In order to ensure that the results we obtained were not confined to HUH7 cells, we transfected R1, R7, and R8 constructs into HeLa and HepG2 cells to assay the constructs in different cell lines. The resultant pattern of CAT activity were qualitatively similar to that observed in Huh7 cells (Fig. 7).

DISCUSSION

HCV is believed to be a distant relative of flaviviruses (Choo *et al.*, 1989, 1990; Han *et al.*, 1991; Houghton *et al.*, 1991), but its genome has structural features at the 5' and 3' termini shared with that of poliovirus (Kitamura *et al.*, 1981) in two respects. First, the 5' UTR of HCV is relatively long, contains multiple ORFs, can be modeled into a highly ordered structure (data not shown), and has a putative hairpin structure at the 5' terminus (Han *et al.*, 1991; Inchauspe *et al.*, 1991; Okamoto *et al.*, 1991, 1992). Second, the 3' UTR of HCV is short and has a homopolymer tail (Han and Houghton, 1992). Since the 5' UTR of poliovirus RNA is translated by a cap-independent internal initiation mechanism (Pelletier and Sonenberg, 1988), we searched for the same mechanism in the 5' UTR of HCV-1 RNA. However, we failed to detect such an activity *in vivo* in conjunction with poliovirus infection. In addition, we could not detect evidence for internal initiation using a dicistronic mRNA approach, a standard DNA transfection system for the detection of IRES in a test RNA (Jang *et al.*, 1989; Pelletier and Sonenberg, 1988). Our results are not cell type-specific, since we obtained similar results from several human cell lines including HeLa and HepG2 cells (data not shown). Taken together, we conclude that the 5' UTR of HCV-1 RNA does not contain an IRES. Furthermore, the fact that all test RNAs having the hairpin at the 5' terminus were translationally inactive strongly suggests that translation of HCV RNA is 5' end-dependent.

In contrast to our results from HCV-1, a group I isolate, Tsukiyama-Kohara *et al.* (1992) recently reported the detection of IRES within the 5' UTR of HCV RNA from two Japanese isolates which belong to group II

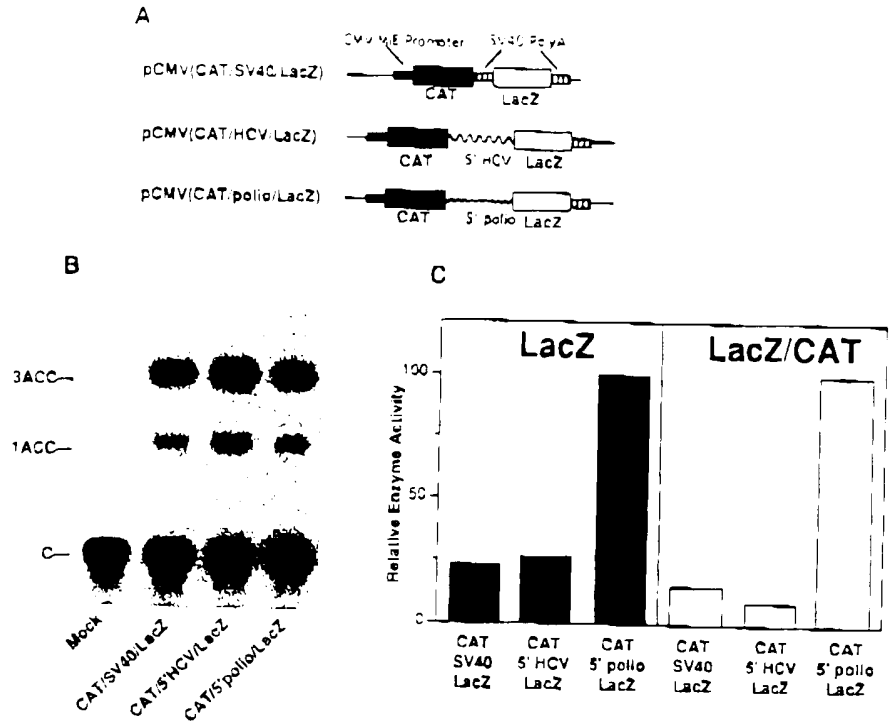

FIG. 6. Expression of CAT and LacZ from dicistronic mRNAs. (A) Schematic representation of expression vectors for each mRNA. In each plasmid, the CMV major immediate-early promoter (MIE) and SV40 polyadenylation signal are indicated. (B) CAT activity from each dicistronic mRNA. (C) Relative LacZ activity normalized to CAT activity.

and III, respectively. Currently, it is difficult to explain these discrepant results. Although it is less likely, it may be formally possible that different groups of HCV have adapted different translation strategies during the course of evolution. The two Japanese HCV isolates vary in the strength of internal initiation by twofold (Tsukiyama-Kohara *et al.*, 1992). The nucleotide sequence in the 5′ UTR of the two reported Japanese HCV isolates differs from each other by 5% and from HCV-1 by 3 and 6%, respectively. Among HCV isolates including these three, the sequence heterogeneity within the 5′ UTR is mainly clustered between nucleotide 200 and 250, which may form a potential secondary structure. In this structure, a pyrimidine track which may be important for internal initiation (Luz and Beck, 1991; Pestova *et al.*, 1991) is located within a loop region (Tsukiyama-Kohara *et al.*, 1992). Although HCV-1 has this sequence in the same region, our results do not indicate that the presence of it confers a cap-independent translation phenotype. Alternatively, *in vitro* systems can be influenced in their cap dependence by levels of K^+ ion in the lysate (Herman, 1987). It is not known what K^+ ion levels were present in the lysates used in the earlier report by Tsukiyama-Kohara *et al.* (1992).

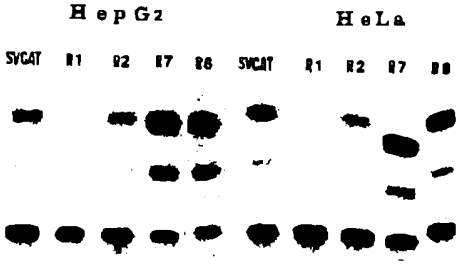

FIG. 7. Translation of hybrid CAT RNAs transfected into HepG2 cells and HeLa cells. RNA transfection and CAT assay were performed as described in Fig. 3 legend.

At the 5′ terminus, HCV-1 RNA has a 27-nucleotide sequence that has a potential to form a hairpin structure with calculated free energy of −14.5 kcal/mol (Han *et al.*, 1991). Although the existence of this hairpin in physiological conditions needs to be verified by nuclease digestion experiments, conservation of its structure, but not the primary sequence, in all putative full-length HCV sequences reported to date (Chen *et al.*, 1992; Han *et al.*, 1991; Inchauspe *et al.*, 1991; Okamoto *et al.*, 1991, 1992) implies that the putative hairpin could be functionally important. We proved that sequence residing within this proposed 5′ hairpin functions as a potent translational inhibitor. It was shown that a stable secondary structure in the 5′ UTR of

mRNA ($\Delta G = -50$ kcal/mol) reduces translation efficiency (Kozak, 1986). By comparison, the putative 5′ hairpin of HCV has a relatively weak secondary structure, which may not be sufficient to block the unwinding activity of the initiation complex (Sonenberg, 1988). However, because of its location, it may block ribosome entry onto the 5′ end of the RNA. Alternatively, it may be a binding site for cellular factor(s) which could be involved in viral replication or encapsidation. RNA hairpins have been implicated in translational control of cellular and viral mRNAs. In ferritin mRNA, a hairpin in the 5′ UTR reversibly binds a cytosolic protein in the absence of iron, which results in translation repression (Rouault *et al.*, 1988). In *Xenopus* oocytes, introduction of an artificial hairpin into the 5′ terminus of a test mRNA results in regulation of translation, which is specific to the stage of differentiation (Fu *et al.*, 1991). A 5′ proximal hairpin is highly conserved between enteroviruses and rhinoviruses. In poliovirus, the RNA hairpin is required for efficient translation and viral replication and mutation in this region significantly lowers translation efficiency, suggesting that the hairpin potentiates the internal ribosome initiation process possibly by its interaction with downstream element or protein factor(s) (Simoes and Sarnow, 1991). Thus the role in translation played by the putative 5′ hairpin of the HCV genome is opposite to that of the poliovirus genome, a marked difference between HCV and poliovirus.

We have located a putative *cis*-acting element that efficiently enhances translation within an 86-nucleotide region, which contains the PEST-IV homology box. The latter 28-nucleotide sequence is nearly perfectly conserved among HCV isolates and shares 90% nucleotide sequence identity with pestiviruses (Han *et al.*, 1991), implying a functional significance. Although we have not determined the precise 5′ and 3′ border of the positive element which enhances translation, we have demonstrated that the PEST-IV homology box is a part of such an element. Currently, it is unknown how this element augments translation *in vivo*. Based on its relatively short sequence requirement, it may facilitate translation by providing a higher relative affinity for limiting component(s) of translation machinery as suggested for alfalfa mosaic virus (Jobling and Gehrke, 1987).

The upstream ORFs, especially the last three of the five, are conserved in all HCV isolates suggesting their possible role in translation. However, defining a role for the first four ORFs in translational control is complicated by two structural features: (1) the first ORF is a part of the 5′ hairpin and (2) three of the remaining four ORFs are overlapping. A deletion in ORF1 (R2) resulted in a moderate increase in CAT activity which may be explained by disruption of the inhibitory element at the 5′ terminus. Removal of 35 nucleotides including the second AUG (R3) resulted in a fourfold increase in CAT activity. Subsequent deletion of the remaining ORFs (R4–R6) did not change CAT activity. Deletion of AUG codon in the fifth ORF (R7) results in a 2.5-fold increase in CAT activity. These data suggest that the upstream ORFs may function as negative modulators of translation. This is consistent with the ribosome scanning hypothesis that has been reported to be operative for the majority of cellular and viral mRNAs (Kozak, 1983, 1989).

We have mapped three putative control elements in the 5′ UTR of HCV-1 RNA. However, the fact that the full-length HCV-1 RNA is translationally inactive raises a question as to how HCV initiates polyprotein synthesis upon infection. In the absence of experimental evidence from a cell culture system, one can consider several possibilities. Perhaps HCV infection primes initial translation by viral component(s). This could either be an HCV-encoded factor(s) which derepresses the translational inhibition imposed by the 5′ repressor element in the genomic RNA or an as yet unidentified *cis*-acting element(s) elsewhere in its genome downstream from polyprotein initiation codon, which allows internal initiation. However, in view of the arrangement of the repressor element at the 5′ end and two putative *cis*-acting elements within the 5′ UTR of HCV, we propose that active mRNA is present in infected cells as a separate entity. Previously, we reported the detection of both 5′ and 3′ subgenomic RNAs (Han *et al.*, 1991). Although the biological significance of these RNAs has not been established, we believe that the 5′ subgenomic RNA could possibly be a viral mRNA based on the fact that a CAT RNA having the 5′ leader of this RNA is translationally active. Currently, the origin of this 5′ subgenomic RNA is unknown. One possibility is that it could be transcribed from a specific promoter element within the 5′ leader by RNA dependent RNA polymerase, for example, as described in Sindbis virus (Strauss and Strauss, 1986).

We speculate that viral protein synthesis in infected cells may be regulated at two or more levels, including mRNA production and control at the level of translation. HCV is believed to exist at low titer in clinical samples. However, HCV infection is persistent and leads to chronic hepatitis and hepatocellular carcinoma at unusually high frequencies (Dienstag and Alter, 1986; Houghton *et al.*, 1991). We hypothesize that viral replication and translation control operating at various levels within the 5′ leader of HCV may be a genetic mechanism, in conjunction with a possible immunologic mechanism (Weiner *et al.*, 1992) for the observed pathobiology of viral infection. Our findings of transla-

tional control elements in HCV RNA provide intriguing prospects for future HCV research.

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EXHIBIT B

Expression and Immune Response to Hepatitis C Virus Core DNA–Based Vaccine Constructs

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Hepatitis C virus (HCV) is a major worldwide cause of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma. The development of vaccines against HCV have been complicated by the high variability of the envelope region, and it is likely that the cellular immune responses to viral structural proteins may be important for eradicating persistent viral infection. Recently, it was reported that the injection into muscle cells of plasmids encoding viral genes resulted in the generation of strong cellular immune responses. We constructed vectors that express the highly conserved HCV core gene. In this regard, the pHCV 2-2 construct contained the entire HCV core region and pHCV 4-2 contained both the 5′ noncoding region and the core gene. Cellular expression of HCV core protein was assessed following transfection into human and murine cell lines, and higher intracellular levels of the 21-kd core protein were observed with pHCV 2-2. These HCV core DNA constructs were used to immunize BALB/c mice and produced low-level anti-HCV core humoral immune responses. To assess cytotoxic T-lymphocyte (CTL) activity generated *in vivo*, a cloned syngeneic SP_2/O myeloma cell line constitutively expressing HCV core protein was established and inoculated into BALB/c mice to produce growth of plasmacytomas. Strong CTL activity was generated because the tumor size and weight in pHCV 2-2–immunized mice were remarkably reduced compared with mice injected with mock DNA. Spontaneous CTL activity was also exhibited by splenocytes in an *in vitro* cytotoxicity assay. These investigations demonstrate that plasmid constructs expressing HCV core protein generate strong CTL activity, as assessed both *in vivo* and *in vitro*, and are promising candidates as antiviral agents. (HEPATOLOGY 1996;24:14-20.)

Hepatitis C virus (HCV) is a positive-strand RNA virus with a linear genome of about 9,500 bases. Different isolates show considerable nucleotide sequence diversity, leading to the subdivision of HCV genomes in at least six genotypes.[1] In all genotypes, the viral RNA contains a large, open reading frame that encodes a polyprotein precursor of 3010 to 3033 amino acids.[2-6] This precursor is cleaved by cellular and viral proteinases to give rise to the core, envelope (E1, E2), and nonstructural proteins (NS2-NS5).[7-9] The coding sequence of the RNA genome is preceded by a 5′ noncoding region of 324 to 341 nucleotides,[10,11] which is highly conserved among all strains of HCV. This noncoding region forms an extensive and stable secondary structure,[12] and serves as an internal ribosomal entry site—essential for efficient cap-independent viral translation—and probably is also necessary for HCV replication.[13-15]

HCV is a major causative agent of posttransfusion hepatitis. More than 50% of acutely infected individuals progress to a chronic carrier state that frequently results in cirrhosis.[16] In addition, HCV infection is an independent risk factor for the development of hepatocellular carcinoma, as shown by the prevalence of anti-HCV antibodies.[17-20] Currently, there is no universal, highly effective therapy of chronic HCV infection. In several studies, it has been shown that the response rate of chronic HCV infection to interferon alfa treatment was as low as 15 to 25%.[21] Other antiviral nucleoside analogs have been shown to be only marginally effective[22] and may be associated with considerable side effects. Therefore, the development of alternative approaches is of major clinical importance.

There is a need to develop new antiviral agents in an attempt to eradicate persistent HCV infection from the liver. In this regard, direct injection of DNA into animals is a novel and promising method for delivering specific antigens for immunization.[15,23-26] This approach has been successfully used to generate protective immunity against influenza virus in mice and chickens, against bovine herpes virus 1 in mice and cattle, and against rabies virus in mice.[27-31] In most cases, strong, yet highly variable, antibody and cytotoxic T-lymphocyte (CTL) responses were associated with control of infection. Indeed, the potential to generate long-lasting memory CTL without using a live vector makes this approach particularly attractive compared with those involving killed-virus vaccines and subunit vaccines. This direct DNA-based gene-therapeutic approach has shown great utility for generating a CTL response that not only protects against acute infection but also may have benefits in eradicating persistent viral infection,[24,26,28-38] of which HCV is an important human prototype. Because the HCV core gene is highly conserved among the various genotypes, we explored its use in a polynucleotide-based vaccine strategy to generate a CTL response in the host.

MATERIALS AND METHODS

Plasmids and Cell Lines. A complementary DNA encoding the HCV core gene was isolated from an anti-HCV–positive individual as described by Wakita et al.[14] This complementary DNA fragment was inserted into a plasmid expression vector containing a rous sarcoma virus enhancer element and driven by a cytomegalovirus promoter. As shown in Fig. 1A, one construct, designated pHCV 2-2, contains the entire HCV core gene, and the other, pHCV 4-2, contains the HCV core coding, as well as the 5′ noncoding region. The con-

Abbreviations: HCV, hepatitis C virus; CTL, cytotoxic T lymphocyte.

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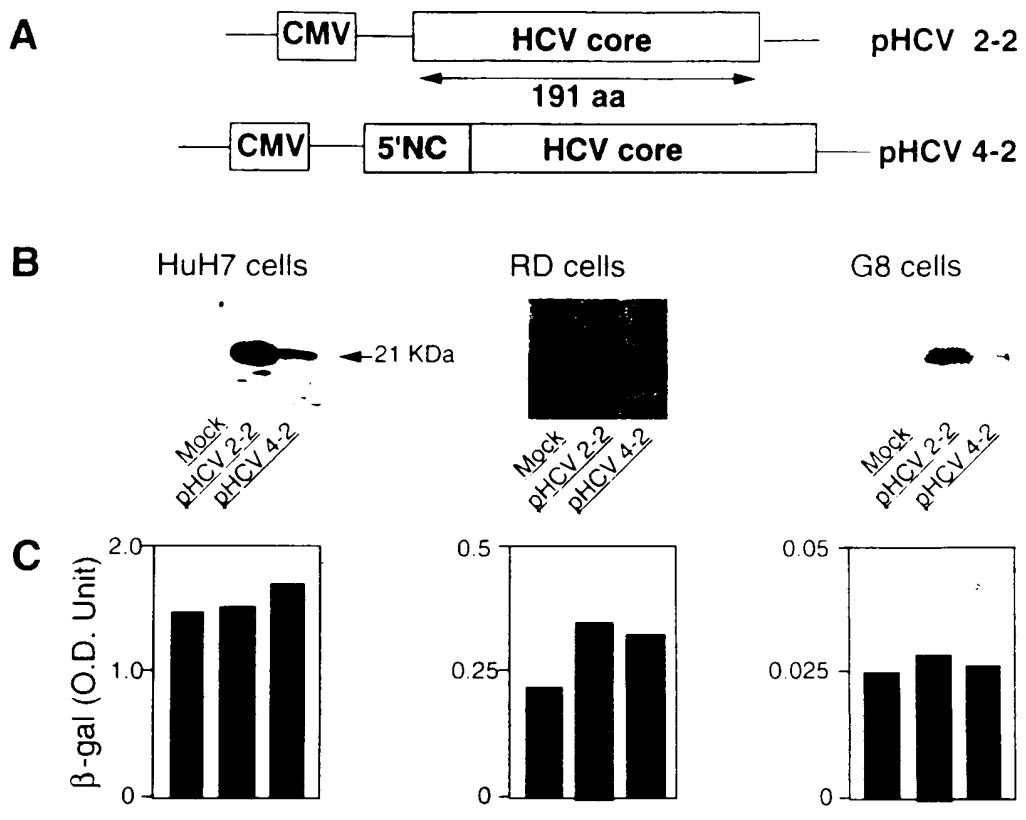

FIG. 1. Intracellular expression of the 21-kd HCV core protein. (A) Cartoon of the HCV core expression vector pHCV 2-2 that contains the entire HCV core gene, and pHCV 4-2 that has both the 5′ noncoding region and core coding region. (B) Western blot analysis demonstrates HCV core gene expression in HuH-7 human hepatocellular carcinoma cells and human (RD) and mouse (G8) muscle cell lines. (C) Transfection efficiency as measured by expression of β-galactosidase after cotransfection with HCV core DNA vaccine constructs at a 4:1 ratio of pHCV 2-2 or 4-2 to pSV-β gal.

structs were grown in DH5α cells, and plasmid DNA was purified by CsCl gradient ultracentrifugation as described.[35]

To assess intracellular levels of HCV core protein following transient transfection of plasmid constructs, HuH-7 cells, a human hepatoma cell line, RD cells, a human rhabdomyosarcoma cell line, and G8 cells, a mouse myoblast cell line, were employed. The SP_2/O syngeneic BALB/c mouse myeloma-derived cell line was used to generate target cells to measure CTL activity both *in vivo* and *in vitro*.[39] All cell lines were obtained from the American Tissue Culture Collection (Rockville, MD).

In Vitro *Studies*. The two HCV core DNA constructs were transfected into HuH-7, RD, and G8 cells by the calcium phosphate precipitation method.[40] Two days after transfection, cells were analyzed for 21-kd core protein expression by Western blot analysis. β-Galactosidase assays were performed following a standard protocol (Promega protocols and applications guide, 2nd ed., 1991, Promega Corp., Madison, WI), which were used to normalize Western blots for transfection efficiency. In brief, cell lysates were prepared in RIPA buffer (0.15 mol/L NaCl, 1% NP-40, 50 mmol/L Tris, 0.5% deoxycholate, and 1% sodium dodecyl sulfate), separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electrotransferred onto Immobilon-P membranes (Millipore, Bedford, MA). After blocking with 3% nonfat dry milk, membranes were incubated with a previously developed anti-HCV core monoclonal antibody, designated C7-50A,[41] followed by detection with a ^{125}I-labeled goat anti-mouse polyclonal antibody (Dupont, New England Nuclear, Billerica, MA). In addition, immunofluorescence staining was performed to determine the cellular localization of the HCV core protein as described previously.[41]

***Plasmid DNA Immunization*.** Pathogen-free BALB/c female mice, aged 6 to 8 weeks, were purchased from Charles River Breeding Laboratory (Boston, MA) and used for all *in vivo* studies. To enhance muscle cell uptake of plasmid DNA, the quadriceps were injected first, at multiple sites with a total of 100 μL of 0.25% bupivacaine. Twenty-four hours after bupivacaine administration, 100 μg of plasmid DNA was injected into the same region at multiple sites. Thereafter, plasmid DNA constructs were injected every 2 weeks for a total of four intramuscular immunizations. Immunizations with recombinant HCV core-glutathione S-transferase fusion protein (1 μg)[42] was performed subcutaneously in complete Freund's adjuvant. BALB/c mice were inoculated every 2 weeks for a total of four immunizations.

***Antibody Assays for HCV Core Antigen*.** To measure HCV core antibodies in serum of immunized mice, the following assay was developed and employed. In brief, a HCV core-GST fusion protein (0.5 μg per well) was used to coat microtiter plates (Falcon, Microtest IIIM Flexible Assay Plate, Pittsburgh, PA). Plates were incubated overnight at 4°C. After blocking with 3% bovine serum albumin in phosphate-buffered saline for 2 hours at 20°C, mouse serum diluted 100-fold was added to the plates and incubated at 20°C for an additional 2 hours. After washing with phosphate-buffered saline containing 0.05% Tween, 1×10^5 cpm of ^{125}I-labeled goat anti-mouse immunoglobulins G and M was added. Following a 1-hour incubation, plates were washed, and radioactivity bound to the plate was determined in a gamma well counter.

***Cytotoxic T-Lymphocyte Activity*.** To assess CTL activity in BALB/c mice, a clonal syngeneic SP_2/O myeloma target cell line was established by stable transfection with a HCV core expression construct; this vector was driven by the elongation 1-α promoter.[43] Stable transfectants were selected in G418-containing medium After cloning by limiting dilution, several SP_2/O cell lines expressing the HCV core protein were established. The presence of HCV core protein was demonstrated by immunofluorescent staining and by using the C7-50 monoclonal antibody. One week after the final immunization with plasmid DNA, 1×10^7 SP_2/O cells expressing the HCV core protein or native SP_2/O cells were inoculated intraperitoneally and subcutaneously into the right and left flank of the mouse. The tumor size and weight, as well as animal survival, were evaluated as an *in vivo* index of CTL activity generated by immunization with the various plasmid DNA constructs.

For *in vitro* studies, CTL activity was measured 1 week following SP_2/O myeloma cell inoculation. Mice were killed and a suspension of spleen cells was prepared from each animal. It is important to emphasize that splenocytes were not prestimulated with HCV core peptides or cells expressing HCV core peptides. Thus, this assay assesses spontaneous CTL activity present in the spleen of immunized mice. Native SP_2/O or SP_2/O cells expressing HCV core antigen were labeled with 100 μCi of ^{51}Cr (Amersham, Arlington Heights, IL) for 2 hours and washed three times with phosphate-buffered saline. CTL activity was determined in a standard 4-hour ^{51}Cr re-

lease assay using U-bottom 96-well plates containing 1×10^4 target cells per well. The percent specific release of ^{51}Cr-labeled target cells was calculated using the following formula: (experimental release − spontaneous release)/(total release − spontaneous release) × 100. Maximum ^{51}Cr release was determined by lysis of target cells with 5% Triton X-100. All assays were performed in triplicate.

Statistical Analysis. To compare differences between two groups, a Student's *t* test was used. *P* values < .05 were considered significant.

RESULTS

Intracellular expression of the 21-kd core protein following transfection with the pHCV 2-2 and pHCV 4-2 constructs was evaluated in HuH-7, RD, and G8 cells by Western blot analysis, as shown in Fig. 1B. Transfection efficiency was assessed by cotransfection with a β-galactosidase–expressing plasmid (Fig. 1C). It was surprising that pHCV 2-2 (Fig. 1B), which contains only the core gene, consistently gave higher intracellular levels of the 21-kd core protein as expressed in HuH-7, RD, and G8 cells compared with pHCV 4-2. There was only a minor difference in transfection efficiency between mock DNA and the two HCV-DNA constructs within the same cell line. Transfection efficiency was highest in HuH-7 cells, followed by the human (RD) and mouse (G8) muscle cell–derived cell lines. As shown in Fig. 2, core protein was found localized to the cytoplasm in cells transfected with the pHCV 2-2 construct. There was no secretion of HCV core protein into the culture supernatant as measured by a two-site monoclonal antibody–based immunoassay.[41]

The anti-HCV core humoral immune response to the various DNA constructs was assessed by measurement of antibodies in serum of mice following four intramuscular immunizations of 100 μg plasmid DNA. As shown in Fig. 3, pHCV 2-2–immunized mice demonstrated a 40% serocoversion rate, whereas only 20% of the mice immunized with pHCV 4-2 developed anti-HCV core antibodies. As a positive control, we compared the anti-HCV core antibody response in mice (n = 5) receiving four injections of recombinant HCV core-GST fusion protein (5 μg), and found that all animals seroconverted and that the anti-HCV core titers were considerably higher than in mice immunized with HCV core DNA constructs (data not shown).

To assess CTL activity *in vivo*, a mouse tumor model was established. As shown in Fig. 4A and B, syngeneic BALB/c-derived SP_2/O myeloma cells were stably transfected with a plasmid construct expressing the HCV core, as well as part of the envelope genes under the control of the elongation factor 1-α promoter. Such cells will express and present endogenously processed HCV core peptides in the context of major histocompatibility complex class I molecules on the cell surface to the immune system. A Western blot of the clonal SP_2/O cell line constitutively expressing the 21-kd HCV core protein is shown in Fig. 4B. Mice injected with this cell line will rapidly develop large plasmacytoma tumors. To maximally "challenge" the level of CTL activity, mice were inoculated with a large tumor (1×10^7 SP_2/O myeloma cells) burden into multiple sites, as illustrated in Fig. 4C. The experimental design involved four inoculations of either 100 μg mock DNA, pHCV 2-2, or pHCV 4-2 every 2 weeks, followed by a tumor cell challenge 1 week after the fourth and last injection. In this model system, if CTL activity is generated, there will be inhibition of tumor growth and increased animal survival rate.[44] Table 1 illustrates the tumor size 1 and 2 weeks after inoculation of 1×10^7 SP_2/O HCV core protein expressing myeloma cells in nonvaccinated mice compared with animals receiving the plasmid DNA (mock), pHCV 2-2, and pHCV 4-2 constructs. At 1 week, all mock DNA-immunized mice developed plasmacytomas at all injection sites. In the pHCV 2-2– or pHCV 4-2–inoculated mice, tumors were found at only 40% and 65% of the injection sites, respectively. Two weeks after challenge with this large SP_2/O myeloma cell burden, the mean tumor size was significantly reduced only in the pHCV 2-2–vaccinated mice. Table 2 demonstrates that the protective effects were specific, because native SP_2/O cells (without HCV core peptide expression) grew rapidly and equally well in all vaccinated mice.

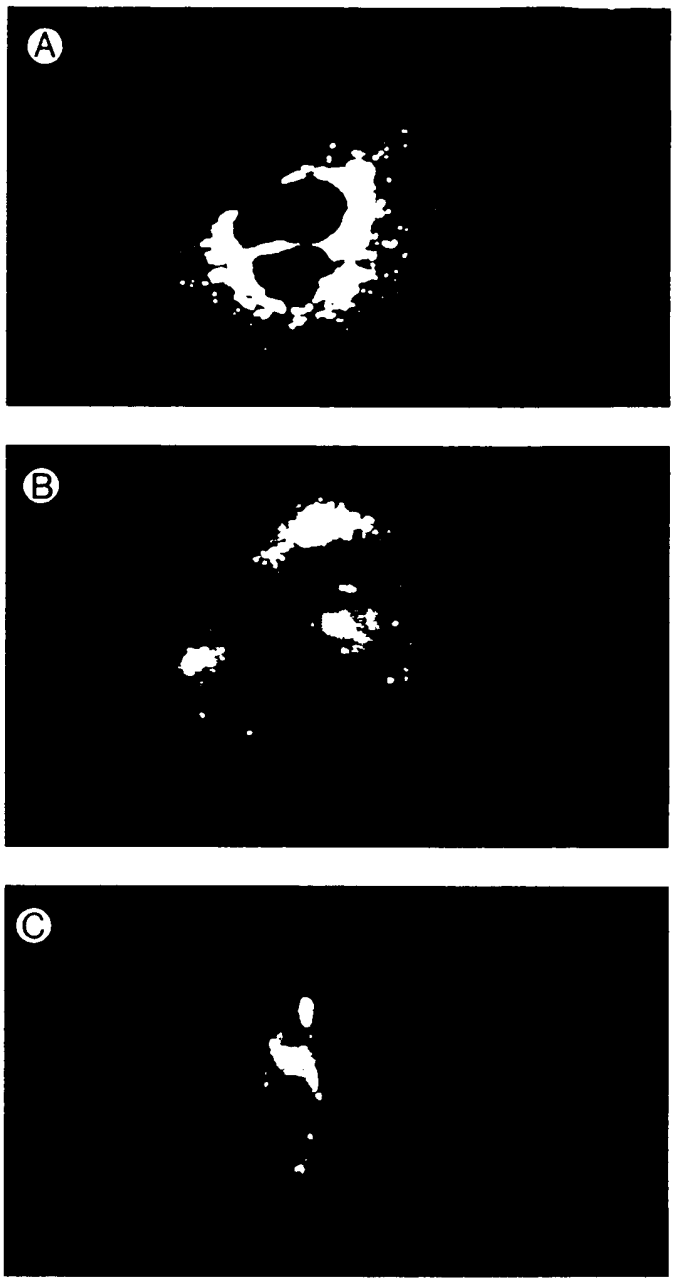

FIG. 2. Immunofluorescent studies of HCV core protein expression as detected by the C7-50 monoclonal antibody. Note the intense cytoplasmic staining of HCV core protein in (A) HuH-7 cells, (B) RD cells, and (C) G8 cells transiently transfected with the pHCV 2-2 construct.

Further support for generation of CTL activity is illustrated by the data presented in Fig. 5 with respect to overall tumor burden (weight) produced at the subcutaneous and intraperitoneal injection sites. Tumor weight was assessed 16 to 18 days after injection of 1×10^7 SP_2/O–HCV core myeloma cells. There was a significant reduction in tumor burden in animals vaccinated with pHCV 2-2. Finally, Fig. 6A shows representative examples of tumors in mice 2 weeks after inoculation with 1×10^7 SP_2/O–HCV core expressing cells. In Fig. 6A, mice immunized with mock DNA (right) are

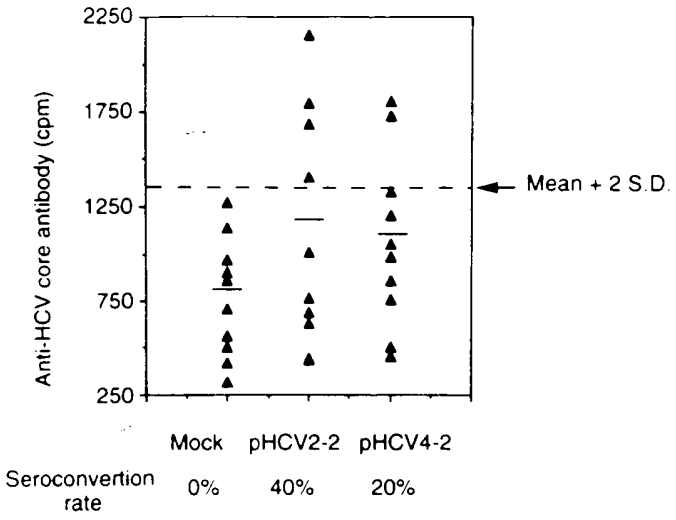

FIG. 3. Humoral immune response following four inoculations with 100 μg of either mock DNA construct or a pHCV 2-2 or pHCV 4-2, as measured by anti-HCV core binding activity in serum. Serum from 10 mice in each group was tested, and a positive result was defined as a value greater than the mean ± 2SD of the mock DNA–immunized control group.

TABLE 1. Tumor Size After Challenge With SP_2/O Cells Expressing HCV Core Antigen in Nonimmunized (normal) or Mice Receiving Intramuscular Injections of Mock DNA, pHCV 2-2, and pHCV 4-2 Constructs

DNA	Tumor Size: Not Detectable	<5 mm	<10 mm	<20 mm	>20 mm	mean ± SD
Normal	0	4	5	1	–	6.70 ± 3.75*†
(n = 5)	0	0	0	4	6	22.50 ± 7.49*
Mock	0	5	10	5	–	7.45 ± 4.18*‡
(n = 10)	0	0	0	5	15	22.50 ± 4.55*
pHCV 2-2	12	5	3	0	–	1.58 ± 2.30
(n = 10)	3	1	5	11	0	10.60 ± 6.63
pHCV 4-2	7	7	6	0	–	3.55 ± 3.12§
(n = 10)	0	0	1	10	7	19.33 ± 7.21
r-HCV	0	2	3	0	–	7.33 ± 2.36*†
(n = 3)	0	0	0	4	2	18.33 ± 7.21

NOTE. The first set of data represent findings at 1 week after tumor injection; the second set represent findings at 2 weeks after tumor injection.

Abbreviation: r-HCV, recombinant HCV core-GST fusion protein.

* $P < .0001$, pHCV 2-2 vs. other groups.

† $P < .05$, ‡ $P < .01$, § $P < .05$, ‖ $P < .01$, pHCV 4-2 vs. other groups.

compared with those vaccinated with pHCV 2-2 (left). Finally, as shown in Fig. 6B, survival rate was enhanced in mice immunized with pHCV 2-2 compared with pHCV 4-2 and mock DNA constructs.

To confirm the generation of CTL activity *in vivo*, experiments were performed with splenocytes *in vitro*. The assay measures spontaneous CTL activity because there was no prior stimulation of cells by HCV peptides. Figure 7 illustrates the CTL activity exhibited by splenocytes derived from mock DNA–, pHCV 2-2–, and pHCV 4-2–immunized mice at effector/target ratios of 50:1 and 100:1 against SP_2/O HCV core expressing cells compared with native SP_2/O target cells. It is noteworthy that splenocytes derived from pHCV 2-2 and pHCV 4-2–immunized mice specifically killed only the core antigen–expressing SP_2/O cells. We found that pHCV 2-2 was more effective in generating CTL activity than the pHCV 4-2 construct.

DISCUSSION

The high mutational rate of the HCV genome may be related to the establishment of persistent viral infection and subsequent disease chronicity.[16,45,46] The cellular immune

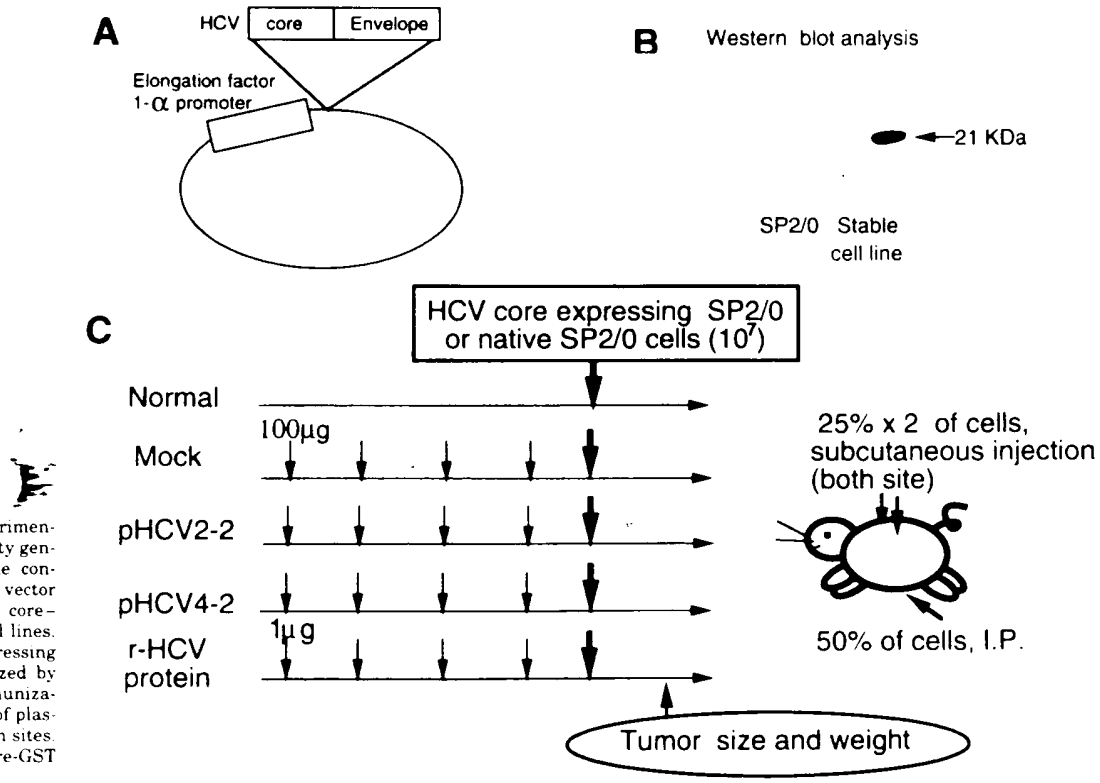

FIG. 4. Cartoon of the experimental design to assess CTL activity generated by the various vaccine constructs *in vivo*. (A) Expression vector used to establish stable HCV core–producing SP_2/O myeloma cell lines. (B) A clonal SP_2/O cell line expressing the 21-kd core protein analyzed by Western blot analysis. (C) Immunization schedule and production of plasmacytomas at various injection sites. r-HCV, recombinant HCV core-GST fusion protein.

TABLE 2. Tumor Size 1 Week Following Injection of Native SP_2O Cells

DNA	Tumor Size: Not Determined	<5 mm	<10 mm	20 mm	mean ± SD*
Mock (n = 5)	1	2	2	3	8.44 ± 4.73
pHCV 2-2 (n = 5)	1	2	3	4	8.80 ± 3.94
r-HCV (n = 3)	1	1	1	1	6.25 ± 3.79

Abbreviation: r-HCV, recombinant HCV core-GST fusion protein.
* There was no significant difference between the three groups.

events involved in liver damage and viral clearance during HCV infection have only partially been defined. In an attempt to examine a potential pathogenic role of liver-infiltrating lymphocytes in patients with chronic HCV infection, Koziel et al. examined the CTL response of such cells and demonstrated a HLA class I–restricted $CD8^+$ T-lymphocyte (CTL) response that was directed against both structural and nonstructural regions of HCV polypeptides.[47,48] Other investigators have also noted the existence of CTLs in peripheral blood mononuclear cell populations that recognize epitopes on core and the other viral-related proteins during chronic HCV infection.[49,50] Botarelli et al.[51] and Ferrari et al.[52] found HLA class II–restricted $CD4^+$ T-cell–mediated proliferative responses to several recombinant proteins derived from different regions of HCV in patients with chronic HCV infection. It is noteworthy that there was a correlation between T-cell responses to HCV core protein and a clinically benign course of the liver disease, as well as subsequent eradication of the virus. However, a similar study by Schupper et al.[53] showed that the proliferative response to HCV core protein did not predict a benign clinical course with respect to the severity of the liver disease.

We have recently studied peripheral blood mononuclear cell responses to a recombinant GST-HCV core fusion protein by evaluating the ability of such cells to produce interferon gamma, correlations were made to different clinical outcomes of HCV infection.[42] Individuals who had received interferon alfa treatment and went into clinical and virological emission had a higher response rate (75%, $P < .05$) to HCV core protein compared with those with ongoing hepatitis who failed therapy (31%). These clinical observations suggested to us that if one could augment the host cellular immune response to HCV core antigenic determinants, then it may be possible to enhance viral clearance during persistent infection.

In this respect, two HCV core DNA constructs were studied. The pHCV 4-2 was investigated, because the 5′ noncoding region is highly conserved among genotypes and contains two internal ribosomal entry sites believed important in the replicative life cycle of the virus.[13,15] However, the results suggest that the cap-dependent translation may be more effective than internal ribosomal entry regarding translation and expression of the HCV core protein in muscle cells and cell lines. Indeed, we are led to believe that the superior humoral and cellular immune response induced by pHCV 2-2 is a reflection of the higher intracellular levels of the 21-kd HCV core protein achieved with this construct. Lagging et al.[54] demonstrated that a HCV core DNA vaccine construct led to the generation of high serum levels of anti-HCV core antibodies in BALB/c mice. These results differ considerably from the findings presented here, because our HCV core DNA constructs produced only weak anti-HCV core antibody responses. Lagging et al. employed crude cell extracts of BALB/c 3T3 cells expressing HCV core protein as an antigen source in their assay system, and the differences between the two studies could be explained on this basis.

Relevant to this issue of polynucleotide-based induction of cellular and humoral immune responses to viral nucleocapsid

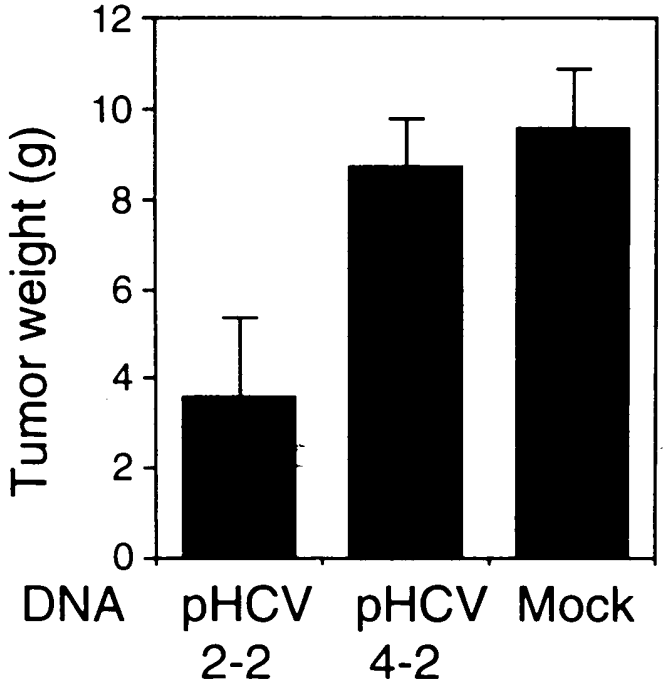

FIG. 5. Illustration of the tumor burden as an *in vivo* index of CTL activity measured 16 to 18 days after inoculation of 1×10^7 SP_2/O–HCV core expressing myeloma cells. Note the striking reduction of tumor weight in mice immunized with the pHCV 2-2 construct ($P < .001$ vs. mock DNA–immunized mice).

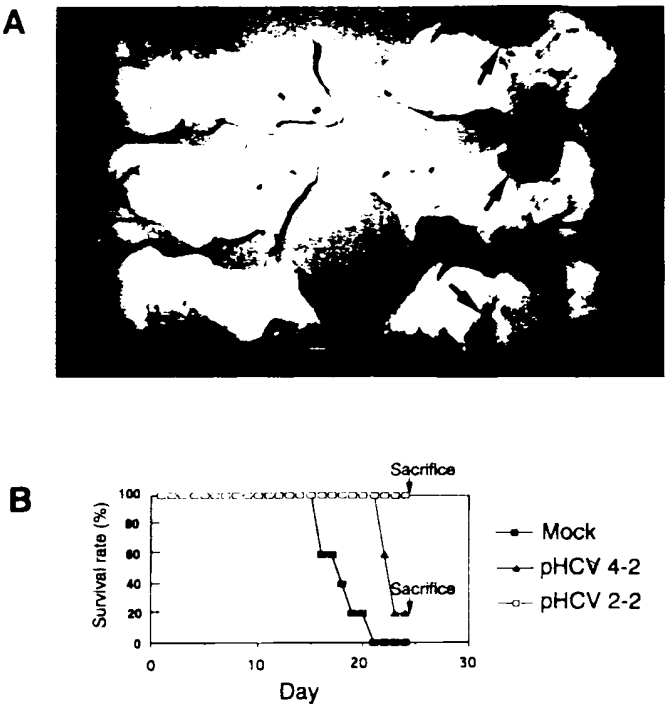

FIG. 6. Effect of immunization on the growth of SP_2/O myeloma cells as an index of CTL activity. (A) Representative examples of tumor growth at 15 days in three mice immunized with pHCV 2-2 (left) compared with mock DNA (right). Note the large size of the tumors (arrows). (B) Survival rate of the vaccinated mice. Note that all mice (n = 10) immunized with pHCV 2-2 survived during the observation period, whereas all mock DNA–immunized controls (n = 10) died by 21 days.

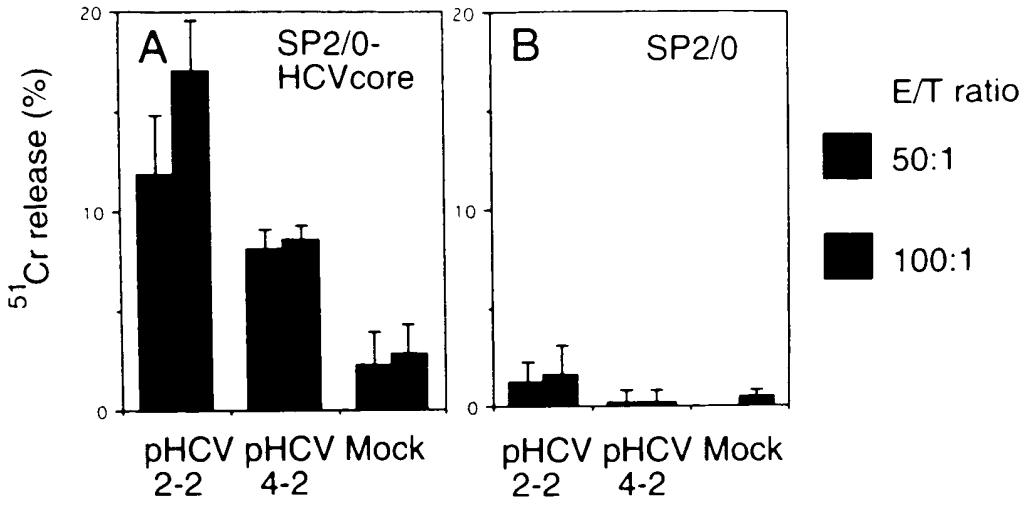

FIG. 7. Specific CTL activity against SP_2/O cells expressing (A) the HCV core antigen compared with (B) native SP_2/O cells. All animals received four immunizations with pHCV 2-2, pHCV 4-2, or mock DNA constructs prior to preparation of splenocyte suspensions.

proteins are the findings with the nucleoprotein of the lymphocytic choriomeningitis virus. Like HCV core, the nucleoprotein of lymphocytic choriomeningitis virus was not secreted when expressed in cells from a transfected plasmid DNA construct. Yokoyama et al.[55] and Martin et al.[36] have reported that a plasmid-based DNA vaccine construct comprised of lymphocytic choriomeningitis virus nucleoprotein induced weak or no humoral immunity in mice following injection into muscle cells, presumably because such cells neither express major histocompatibility complex class II antigens nor stimulate $CD4^+$ helper T and B cells by secretion from the cell. It is likely that only a small fraction of plasmid DNA is taken up and expressed by antigen-presenting cells residing in and around muscle tissue. Therefore, it is reasonable to postulate that the humoral immune response induced by HCV core DNA immunization was weak because of the fact that the core protein is not secreted from the cell to be processed by antigen-presenting cells.[56]

The recent study by Lagging et al.[54] also demonstrated that a HCV core DNA vaccine construct generated CTL activity against HCV core epitopes *in vitro*. However, in this study, it was necessary to use spleen cells derived from immunized mice that were previously stimulated by cells infected with vaccina virus encoding the HCV core gene to demonstrate such CTL activity. We found that the pHCV 2-2 construct generated substantial CTL activity in all mice immunized with this construct, because tumor cell growth was inhibited and the animal survival rate was improved. Thus, CTL activity was highly operative *in vivo* against tumor cells in this animal model system. Furthermore, spontaneous CTL activity was observed *in vitro* as well, because SP_2/O cells expressing HCV core epitope were specifically killed by splenocytes derived from HCV core–immunized mice. Regarding the comparison between pHCV 2-2 and 4-2 constructs, pHCV 2-2 was more effective in generating CTL activity than pHCV 4-2. It is reasonable to postulate that the large amount of core protein produced intracellularly by the pHCV 2-2 construct induced CTL more easily than the smaller amount produced by pHCV 4-2.

It was of interest to determine whether the HCV-DNA constructs would improve the survival rate of mice after tumor challenge. In the mock DNA–immunized normal mice, all died by 21 days after tumor injection (mean survival, 18 days). In contrast, all mice immunized with pHCV 2-2 survived over the observation period, suggesting that CTL activity was present in all pHCV 2-2–immunized animals. Previous studies have also demonstrated that DNA-based immunization may improve animal survival rate in a tumor model. For example, Wang et al.[44] reported that, in human immunodeficiency virus–DNA–immunized mice, at 16 weeks, 90% were completely protected against tumor formation induced by SP_2/O cells expressing the gp160 protein of human immunodeficiency virus. In our studies, the tumors derived from the pHCV 2-2–immunized mice were small and grew slowly, but eventually developed in all HCV core–vaccinated animals. One difference may possibly be caused by the tumor load. Wang et al. challenged mice with 3×10^6 SP_2/O cells, but, in the present investigation, we used 1×10^7 SP_2/O cells.

Our studies suggest that strong CTL activity may be generated to a nonsecreted HCV core protein both *in vitro* and *in vivo*, and that the pHCV 2-2 construct is a promising antiviral agent to test as a therapeutic agent during persistent HCV infection. Finally, polynucleotide vaccination with such a DNA construct may also have value as an immunization approach for prevention of acute HCV infection as well.

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EXHIBIT C

Genetic Immunization Generates Cellular and Humoral Immune Responses Against the Nonstructural Proteins of the Hepatitis C Virus in a Murine Model[1]

Jens Encke, Jasper zu Putlitz, Michael Geissler, and Jack R. Wands[2]

Exposure to hepatitis C virus (HCV) is associated with a high prevalence of persistent viral infection and the development of chronic liver disease and hepatocellular carcinoma. Recovery from acute infection may depend upon the generation of broad-based cellular immune responses to viral structural and nonstructural proteins. We used the DNA-based immunization approach in BALB/c mice to determine whether the HCV nonstructural proteins NS3, NS4, and NS5 will induce Ab responses, $CD4^+$ Th cell proliferation, and cytokine release in response to stimulation by recombinant proteins as well as generate $CD8^+$ CTL activity both in vitro and in vivo. We found that the nonstructural proteins were particularly good immunogens and produced cellular immune responses when administered as a DNA construct. Indeed, a tumor model was established following inoculation of syngenic SP2/0 cells stably transfected with NS5. We observed protection against tumor formation and growth only in mice immunized with the NS5-encoding DNA construct, establishing the generation of significant CTL activity in vivo by this technique. The results indicate that genetic immunization may define the cellular immune response of the host to HCV nonstructural proteins and is a promising approach for vaccine development. *The Journal of Immunology,* **1998, 161: 4917–4923.**

Hepatitis C virus (HCV)[3] is the major cause of posttransfusion and sporadic non-A, non-B hepatitis (1) and is found throughout the world. There is a prevalence of 0.6 to 2% in western countries and ≤15% in other regions of the world (2). Approximately 60% of individuals exposed to HCV will develop chronic infection and hepatitis; 20 to 40% will eventually progress to cirrhosis and liver failure (3). More important, persistent HCV infection is associated with a high risk of primary hepatocellular carcinoma, particularly in the setting of hepatic fibrosis and cirrhosis (4). Effective therapy of chronic HCV infection has been limited, at best, and only IFN and ribavirin have been shown to exhibit beneficial antiviral activity (5). Indeed, ~10 to 15% of individuals treated with IFN alone will respond and eradicate HCV from the liver. However, recent studies have revealed that individuals who recover from acute HCV infection develop substantial $CD4^+$ T cell proliferative responses against the nonstructural proteins as compared with those individuals who develop persistent HCV infection (6, 7). This type of cellular immune response suggests that the nonstructural proteins may be the more critical immunogens to eradicate persistent viral infection from the host. In this context, direct injection of DNA encoding for viral genes in combination with different facilitators into the muscle or skin has been shown to induce broad-based humoral and, more important, cell-mediated immune responses, and is especially effective in generating protective cytotoxic T cell responses against a variety of pathogens (8–11). However, the generation of such protective immune responses in humans remains to be established.

In the present investigation, we evaluated in vitro and in vivo humoral and cellular immune responses generated by DNA-based immunization against the three different nonstructural proteins of HCV in a murine model. It was found that the cDNAs encoding for the NS3 serine protease and helicase and NS5 RNA-dependent RNA polymerase were particularly effective in generating high-level $CD4^+$ and $CD8^+$ activities against epitopes that reside on these nonstructural proteins.

Materials and Methods

Plasmid construction

As a source of viral genes, a plasmid designated pBRTM/HCV1-3011 covering the full-length open reading frame (ORF) of HCV was used to clone into expression vectors (12). Constructs pAp031-NS3, pAp031-NS4, and pAp031-NS5 were PCR-cloned after inserting engineered start and stop codons as well as restriction enzyme sites using the following primers: for NS3, 5′-GG TCT AGA TTG ATG GCG CCC ATC ACG GC-3′ (*Xba*I), 5′ CAC ACG CGT TCA CGT GAC GAC CTC CAG GT 3′ (*Mlu*I); for NS4, 5′-G GTC TAG ATG AGC ACC TGG GTG CTC-3′ (*Xba*I) and 5′-CCA GGA TCC TCA GCA TGG AGT GGT ACA-3′ (*Bam*HI); and for NS5, 5′-T CAG TCT AGA ATG TCC GGC TCC TGG CTA AGG GA-3′ (*Xba*I) and 5′-A GCT ACG CGT TCA CCG GTT GGG GAG GAG GT-3′ (*Mlu*I). After PCR amplification using a high-fidelity PCR system (Boehringer Mannheim, Indianapolis, IN), the cDNA fragments were inserted into the plasmid expression vector pAp031 containing a Rous sarcoma virus enhancer element and a CMV promoter (Apollon, Malvern, PA). Constructs were transformed into DH5α cells, and plasmid DNA was subsequently purified by either 2× cesium chloride centrifugation or with a Qiagen Giga kit using the Endofree buffer system (Santa Clara, CA). Correct insertion of cDNAs coding for of the nonstructural proteins was verified by sequencing analysis using standard methods. To establish stable NS3-, NS4-, and NS5-expressing cell lines as target cells for the CTL assays, the nonstructural protein-encoding gene fragments were also cloned into the pcDNA3 and pcDNA3.1 Zeo(−) expression vectors (Invitrogen, San Diego, CA) with a neomycin selectable marker. An *Xba*I and *Mlu*I

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[3] Abbreviations used in this paper: HCV, hepatitis C virus; HBsAg, hepatitis B virus surface Ag; ORF, open reading frame.

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fragment of NS3 and NS5 was subcloned into the *NheI MluI* site of Litmus-38 vector (New England Biolabs, Beverly, MA), cut with *Eco*RI and *Sal*I, and ligated into the *Eco*RI *Xho*I multiple cloning site of pcDNA3 and pcDNA3.1 Zeo(−), respectively. An *Xba*I and *Bam*HI fragment containing NS4 was ligated into Litmus-29 (New England Biolabs), recut with *Kpn*I and *Eco*RI, and subsequently ligated into the pcDNA3 vector. Plasmids were designated pcDNA3-NS3, pcDNA3-NS4, and pcDNA3.1 Zeo(−)-NS5.

In vitro expression

The HuH-7 human hepatoma cell line was transiently transfected with the various constructs by the calcium phosphate method to assess expression levels of HCV nonstructural proteins. In brief, cell lysates were prepared in modified RIPA buffer (0.15 M NaCl, 1% Nonidet P-40, 50 mM Tris, 0.5% deoxycholate, and 1% SDS) after metabolic labeling with [^{35}S]methionine and cysteine for 4 h. Cell lysates were precleared with horse serum and subsequently bound to Sepharose A by preincubation overnight with polyclonal antisera WU 110 (NS3), WU 148/151 (NS4), and WU 115 (NS5) (12). After separating the proteins by SDS-PAGE, the gels were dried and exposed. NS5 protein expression was also determined by Western blot and immunofluorescence analysis using a murine mAb (Biogenesis, Sandown, NH). To generate stably transfected cell lines expressing NS3, NS4, and NS5, the syngenic BALB/c mouse myeloma derived cell line SP2/0 was transfected by electroporation with pcDNA3-NS3, pcDNA3-NS4, or pcDNA3.1 Zeo(−)-NS5. Cells growing in selection medium were cloned by limiting dilution (0.3 cell/well) and screened by the methods described above. However, attempts to clone stable NS4-expressing cell lines were unsuccessful.

Immunization protocol

Female BALB/c (H-2^d) mice were maintained under standard pathogen-free conditions in the animal facility of the Massachusetts General Hospital. Mice were obtained from Charles River Laboratories (Wilmington, MA) and used at the age of 6 to 20 wk for the in vivo studies. A total of 100 μg of plasmid DNA in 100 μl of 0.9% NaCl was injected two and three times over five different sites into the quadriceps muscle of the mice. Booster injections were given into the opposite leg every 14 days. As a positive control for all immunologic experiments, 5 μg of recombinant NS3, NS4, and NS5 protein (Mikrogen, Munich, Germany) was injected i.p. in CFA at day 0 and boosted with the same amount of protein in 0.05% SDS after 4 and 8 wk. As negative controls for these experiments, empty plasmid vector and recombinant hepatitis B virus surface Ag (HBsAg) (Engerix, Smith Kline Beecham, Philadelphia, PA) were employed. All mice were sacrificed at 10 days after the last immunization.

Measurement of humoral immune responses

Levels of anti-NS3, NS4, and NS5 Abs were determined in the serum of each immunized animal by ELISA. In brief, microtiter plates (Microtest IIIM flexible assay plate, Falcon, Oxnard, CA) were coated with the above-described recombinant proteins overnight at 4°C (0.5 μg/well). After blocking with FBS for 2 h at 20°C, a 1/50 dilution of mouse serum was added to the plates and incubated at 20°C for an additional 1 h. After washing four times with PBS containing 0.05% Tween-20, a horseradish peroxidase-conjugated anti-mouse Ab (Amersham, Arlington Heights, IL) was applied at a 1/2000 dilution. Plates were washed following a 1-h incubation, and substrate was added for color development and read in an automatic reader.

Lymphoproliferation and cytokine release assays

Mice were anesthetized with isoflurane (Aerrane, Anaquest, NJ), and spleen cells were harvested. E were removed by incubation in 0.83% NH_4Cl/0.17 M Tris (pH 7.4) for 5 min at 25°C. Spleen cells were washed two times and cultured in triplicate using 96-well round-bottom plates at 5×10^5 cells/well in 200 μl DMEM (Cellgro Mediatech, Washington, DC) containing 10% FBS and 2-ME. Cells were stimulated with recombinant nonstructural proteins NS3, NS4, and NS5-4 at different concentrations (0, 0.01, 0.1, and 1 μg/ml). As negative controls, effector cells were stimulated with recombinant HCV core or HBsAg proteins at the same concentrations. After stimulation for 3 days, [^{3}H]thymidine was added (1 μCi/well). Cells were incubated for an additional 18 h, and the [^{3}H]thymidine incorporation into DNA was measured after harvesting. Incorporation of radioactivity was corrected for background activity (Δ cpm). For determination of cytokine release, effector cells were cultured as described above; IL-2, IL-4, and IFN-γ levels were measured in the culture supernatant by commercial kits according to the manufacturer's instructions (Endogen, Boston, MA).

CTL activity

Spleen cells derived from immunized mice were suspended in DMEM supplemented with 10% FCS and 2-ME (5×10^{-5} M) and analyzed for cytotoxic activity following 5 days of in vitro stimulation. In vitro stimulation was performed in 25-ml flasks with a total volume of 12 ml. Murine rIL-2 was added once at a concentration of 5 U/ml, and responder cells (4×10^7) were cocultured with 2×10^6 irradiated (10,000 rad) syngenic SP2/0 cells stably expressing either the full-length NS3 or NS5 protein (SP2/NS3-3, SP2/NS5-21). After 5 days, cytotoxic effector lymphocyte populations were harvested and washed in serum-free medium; a 4-h ^{51}Cr release assay was performed in 96-well round-bottom plates (total volume of 140 μl) using ^{51}Cr-labeled SP2/NS3-3 or SP2/NS5-21. These cells (1×10^6) were incubated for 1 h with 100 μl of ^{51}Cr (1 mCi/ml) and subsequently washed three times in DMEM containing 10% FCS (4°C). Parental SP2/0 or SP2/19 cells expressing the HCV core protein were used as controls for Ag specificity of lysis and background activity. Assays for CTL activity were performed at lymphocyte E:T ratios of 100:1, 30:1, 10:1, and 3:1, respectively, using 1×10^4 ^{51}Cr-labeled target cells/well. T cell depletion experiments were conducted by incubating effector cells with either an anti-CD4$^+$ or CD8$^+$ mAb containing hybridoma supernatant (GK1.5 anti-CD4, rat or 3.155 anti-CD8, rat) for 30 min at 4°C; next, the cells were washed and then incubated at 37°C with complement (1:5 dilution of low-toxicity rabbit complement; Cedarlane Laboratories, Hornby, Canada) before performing the CTL assay described above.

Assessment of CTL activity in vivo

Mice were immunized i.m. three times with either Mock DNA or pApNS5 vector. Some animals were also immunized i.p with recombinant NS5 protein or a combination of both. Recombinant proteins (5 μg i.p.) were administered as a mixture of NS5-4 (aa 2622–2868) and NS5-12 (aa 2007–2268). At 1 wk after the last immunization with the various plasmid constructs or recombinant protein, 2×10^6 syngenic SP2/0-derived cells stably expressing NS5 were washed, resuspended in 200 μl PBS, and inoculated s.c. into the right flank. SP2/0 cells that stably expressed HCV core protein (SP2/19) were used as a control in selected animals. Tumor formation was assessed at 15 days postinoculation, and the number of animals with tumors and tumor weight was determined.

Results

Expression of HCV nonstructural proteins in mammalian cells

HCV is a positive-strand RNA virus with a genome length of ~9.5 kb. One large ORF encodes for a polyprotein precursor of ~3000 aa that is processed by a combination of host and viral proteases into ≥10 different structural and nonstructural proteins (12–14). We cloned the genes encoding for the individual nonstructural proteins with engineered start and stop codons into an expression plasmid driven by a CMV promoter and a Rous sarcoma virus enhancer (pAp031). The expression vector pcDNA3 containing a neomycin selection marker was also used to generate stable SP2/0-derived cell lines (Fig. 1*a*). The plasmid constructs were sequenced across the cDNA inserts, and protein expression was analyzed in vitro in HuH-7 cells after transient transfection and in SP2/0 target cells after stable transfection, respectively. Signals corresponding to proteins with molecular masses of ~70 kDa for NS3, 30 kDa for NS4, and 125 kDa for NS5 were observed in cellular lysates but not in supernatant from transfected cells (Fig. 1*b*).

Humoral immune responses

Specific Ab responses directed against all three nonstructural proteins were found in all animals by ELISA following three immunizations. No Ag-specific immune responses were detected in mice immunized with mock DNA (Fig. 2*a*). As positive controls, mice were vaccinated three times i.p. with recombinant NS3, NS4, and NS5 proteins in combination with CFA; as expected, the mice demonstrated a strong humoral immune response (data not shown).

Cellular immune responses

To investigate cell-mediated immune responses to the nonstructural proteins, spleen cells were harvested and restimulated either

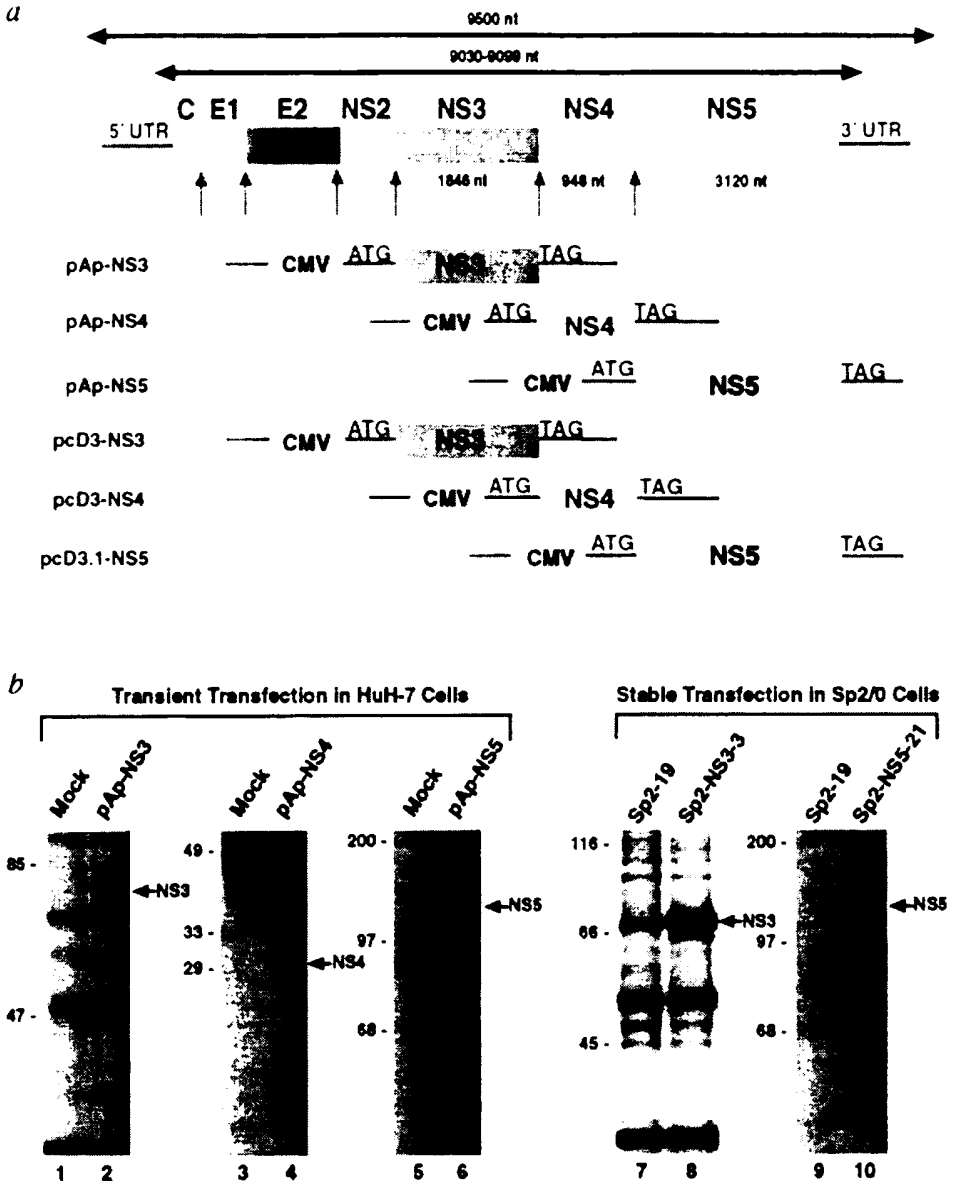

FIGURE 1. Expression of nonstructural proteins following transient transfection of HuH-7 and stable transfection of SP2/0 cells. *a*, a single large ORF of HCV encodes for a polyprotein precursor of ~3011–3030 aa that is cleaved by host signal and virus proteases into the different structural and nonstructural proteins (arrows). Gene sequences of NS3, NS4, and NS5 were PCR-amplified, inserted into pcDNA3 or pcDNA3.1(−), and sequenced. *b*, *lanes 1–6*: After transient transfection of HuH-7 cells with these constructs and controlling for transfection efficiency with a β-galactosidase assay, cells were starved for 30 min in methionine and cysteine-free medium and labeled for 4 h with [^{35}S]methionine and cysteine. Cell lysates were immunoprecipitated with polyclonal rabbit sera specific for the nonstructural proteins, captured by Sepharose A beads, and analyzed by SDS-PAGE followed by autoradiography. *Lanes 1*, *3*, and *5* are mock DNA-transfected cells and serve as negative controls (Mock). *Lanes 2*, *4*, and *6* show specific bands of ~70 kDa for NS3, ~30 kDa for NS4, and 125 kDa for NS5. *Lanes 7–10*: SP2/0 cells were transfected with pcDNA3-based constructs containing the genes for NS3, NS4, and NS5. After antibiotic selection, cells were cloned by limiting dilution (0.3 cells/well), and expanded and analyzed either by radioactive labeling and immunoprecipitation for NS3 or by Western blot for NS5 as described above. *Lanes 7* and *9* represent cell lysates derived from cells stably expressing HCV core protein as a negative control (SP2/19); *Lanes 8* and *10* indicate a specific expression of NS3 and NS5. These cells were used for in vitro stimulation and as target cells in the CTL assays.

with recombinant Ag or with Ag expressed by stably transfected cell lines in vitro. Substantial lymphocyte proliferation was induced by all nonstructural proteins at different Ag concentrations as measured by [^{3}H]thymidine incorporation (Fig. 2*b*). Immunization with recombinant protein i.p. as a means of generating maximum stimulation produced a 5- to 10-fold higher lymphocyte proliferative rate for all three proteins (data not shown). The cytokine profile determined after DNA-based immunization demonstrated a classic Th1 response, with high levels of IFN-γ (Fig. 2*c*) and IL-2 (Fig. 2*d*) secreted into the cell culture supernatant. The cytokine release after incubation with recombinant NS3 could only be studied at a concentration of 0.1 μg/ml, since higher concentrations of NS3 (1 μg/ml) were toxic to the cells. In contrast, very little IL-4 production was observed after genetic immunization with genes encoding for the HCV nonstructural proteins (Fig. 2*e*).

Because CTL responses are essential to eliminate virus from infected cells, we studied the ability of splenocytes derived from immunized mice to lyse syngenic SP2/0 murine myeloma target cells stably expressing NS3 and NS5 proteins in a ^{51}Cr release assay. The NS3- and NS5-immunized mice exhibited a specific

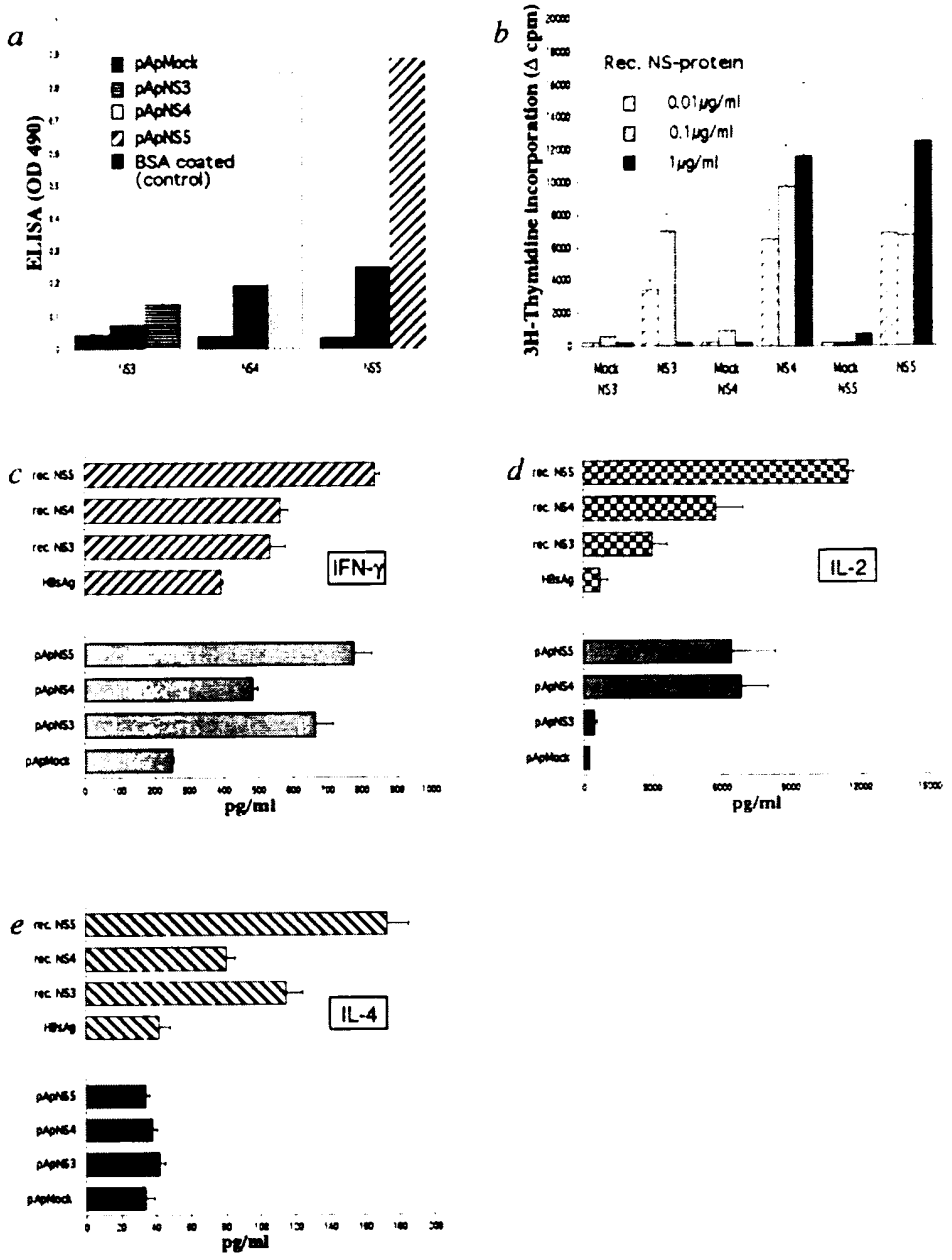

FIGURE 2. *a*, Humoral immune responses to NS3, NS4, and NS5 generated by DNA-based immunization. Serum Ab levels were measured by ELISA (each group: $n = 5$). Controls included wells coated with BSA and sera derived from mock-immunized mice. As positive controls, mice were immunized i.p. with recombinant proteins (data not shown). *b*, T cell proliferation was measured at 3 days after in vitro stimulation with recombinant proteins. Cells were incubated with [^{3}H]thymidine for 18 h and harvested. The Δ cpm was determined by subtracting background activity (i.e., incubation without Ag). Note that incubation of cells with 1 µg of recombinant NS3 protein was toxic; therefore, no proliferation was observed. Mice immunized with recombinant protein in conjunction with CFA had a 5- to 10-fold higher response (data not shown). *c–e*, Cytokine secretion into the supernatant measured after 48 h of in vitro stimulation for IFN-γ (*c*), IL-2 (*d*), and IL-4 (*e*). Note that stimulation with NS3 was performed at 0.1 µg/ml due to toxic effects at 1 µg/ml. For comparison, results are shown for mice that were immunized three times i.p. with recombinant proteins ($n = 4$). As a negative control, mice were immunized with recombinant HBsAg.

cytotoxic T cell response after 5 days of in vitro stimulation, whereas low activity was observed against SP2/0 or SP2/19 (stably expressing HCV core protein) cells used as controls for target cell specificity (Fig. 3, *a* and *b*). To demonstrate the phenotype of cells producing the specific lysis, splenocytes were incubated with $CD8^+$- or $CD4^+$-specific mAbs in the presence of complement. These studies revealed that the cytotoxic activity was mediated by $CD8^+$ cells (Fig. 3*c*). We were unable to establish SP2/0 cell lines stably expressing NS4 protein; therefore, CTL activity was not measured against this HCV nonstructural protein.

In vivo CTL activity was assessed by a tumor model. Only 40% of mice immunized with a cDNA encoding for NS5 protein and challenged with a syngenic murine myeloma cell line (SP2/NS5-21) stably expressing NS5 developed tumors after 15 days. Moreover, tumor size was significantly less ($p < 0.0001$) as determined by the measurement of tumor weight when compared with mice immunized with mock DNA or recombinant NS5 protein or mice immunized with the same syngenic SP2/0 cell line expressing a different HCV structural protein (HCV core) as a control (Fig. 4, *a* and *b*). Indeed, 90 to 100% of mice immunized with mock DNA or challenged with SP2/9 cells demonstrated tumor formation, confirming the specificity of the CTL activity in this tumor model. It is important to emphasize that immunization with recombinant NS5 protein in CFA did not protect animals against tumor formation. To assess the effect of a combination of DNA-based immunization and recombinant protein vaccination, one group of animals was immunized with both. There was partial but significant ($p < 0.03$) protection against tumor formation, but combined immunization was not as effective as immunization three times with a DNA construct encoding for NS5 protein alone (Fig. 4*a*).

Discussion

During active HCV infection, humoral and cellular immune responses have been shown to be polyclonal and multispecific. It is likely that the host immune response produced during persistent

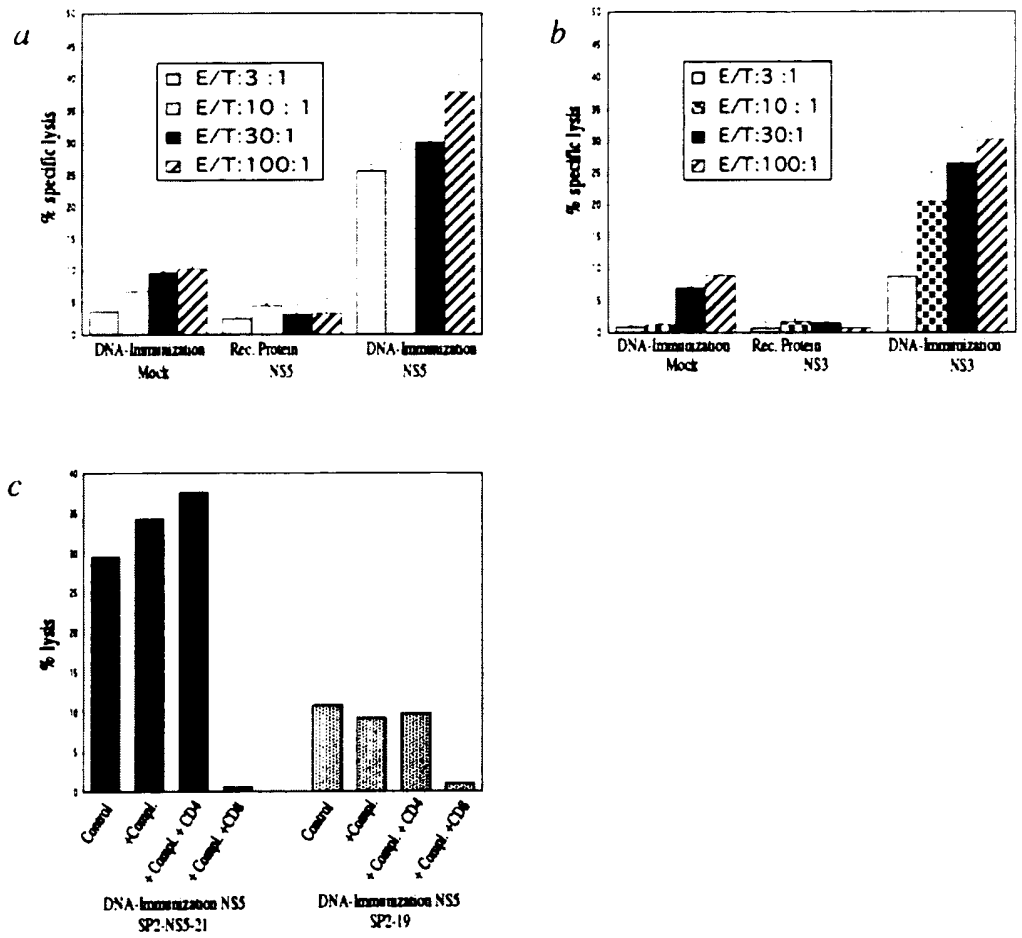

FIGURE 3. Cytotoxic T cell response to NS5 (*a*) and NS3 (*b*) at different E:T cell ratios (100:1, 30:1, 10:1, and 3:1). Splenocytes were incubated in vitro with irradiated murine myeloma cells stably expressing NS3 and NS5 for 5 days ($n = 5$). Subsequently, CTL activity was determined in a 4-h ^{51}Cr release assay against the stable target cell lines. Background activity against SP2/0 or SP2/19 (expressing HCV core) was subtracted to obtain specific lysis values. *c*, In T cell-depletion experiments ($n = 3$), cells were incubated for 30 min on ice with anti-$CD8^+$ or $CD4^+$ mAbs followed by a 30-min incubation at 37°C with complement. Control cells were incubated without complement and anti-$CD8^+$ or $CD4^+$ mAbs. Background activity was determined using SP2/19 cells as a nonrelevant negative control cell line.

HCV infection is responsible, in part, for production of the liver cell injury. However, it may not be sufficiently broad-based or vigorous enough to promote viral clearance and generate protective immunity in individuals with chronic HCV infection (15). Individuals who have recovered from acute HCV infection have recently been shown to develop strong proliferative $CD4^+$ T cell responses directed against the nonstructural proteins (6, 7). More important, the generation of HCV-specific CTL activity appears to be associated with control of viral replication in individuals with chronic hepatitis (16, 17).

It is not known whether the nonstructural proteins NS3, NS4, and NS5 are sufficiently immunogenic to generate broad-based and vigorous CTL responses in vivo. The genetic immunization approach was employed to test this hypothesis, since this technique has been shown previously to induce cellular immune responses of different levels against a variety of pathogens in animal model systems (9–11, 18, 19). The advantage of this method compared with immunizations with soluble recombinant proteins or peptides is its ability to induce a more Th1-like immune response with the production of inflammatory $CD4^+$ T cell as well as cytotoxic T cell activity, presumably due to the intracellular processing of viral proteins into peptides and subsequent loading onto MHC class I molecules in transfected muscle cells as well as to yet to be defined interactions of the complex with APCs. In contrast, immunization with soluble protein primarily leads to a humoral immune response due to processing through the MHC class II pathway. Immunization with synthetic peptides has several disadvantages, since only a limited number of epitopes are available for stimulation of the host immune response. In contrast, all naturally occurring B and T cell epitopes encoded for each protein by the DNA construct of interest are presumably preserved for recognition by TCRs and consequently will generate very broad-based humoral and cellular immune responses (20).

During active viral replication, HCV has a very high mutation rate, and several genotypes and subtypes have been described previously (13, 14). In this regard, the Ags are processed intracellularly in infected hepatocytes, and a large number of epitopes are presented to the immune system. However, neutralizing Abs generated against the envelope region of HCV have been found to be insufficient to provide protection and tend to promote immunoselection of quasispecies (21). In this study, we present evidence that DNA-based vaccination with plasmids encoding for three different nonstructural proteins of HCV is capable of eliciting Ag-specific immune responses in both effector pathways of the immune system. It was noteworthy that all animals developed detectable Ab responses after three immunizations. In this regard,

a

	Immunization		Tumor Challenge	Tumor Formation	Tumor weight (in g +/- SEM)
A	100 μg Mock	3 x i.m.	SP/2NS5-21	10 / 10 (100%)	1.9 +/- 0.2
B	100 μg pApNS5	3 x i.m.	SP/2NS5-21	8 / 20 (40%)	0.7 +/- 0.1
C	100 μg pApNS5	3 x i.m.	SP/2-19	9 / 10 (90%)	2.2 +/- 0.5
D	5 μg recomb Protein	3 x i.p.	SP/2NS5-21	10 / 10 (100%)	1.9 +/- 0.2
E	100 μg pApNS5 5 μg recomb. Protein	2 x i.m. 1 x i.p.	SP/2NS5-21	7 / 10 (70%)	1.1 +/- 0.2

b

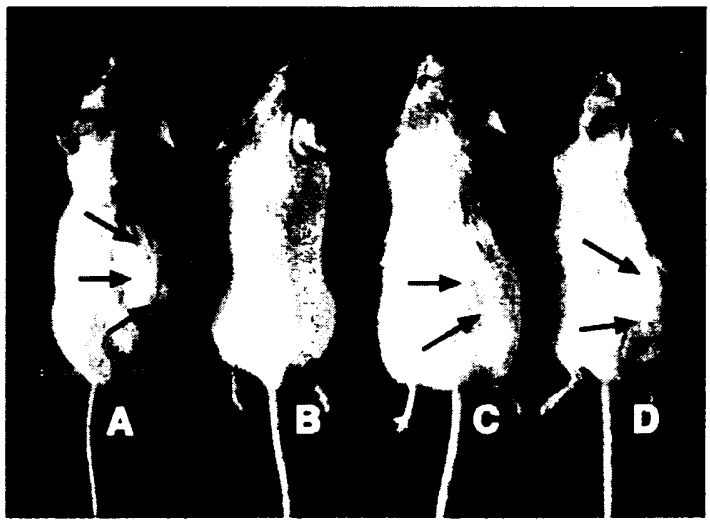

FIGURE 4. *a*, Tumor model to assess CTL activity generated in vivo. Mice were immunized three times with either pApNS5 or a mock DNA (100 μg) or recombinant NS5 protein (5 μg). The final group (E) received a combination of DNA immunization and recombinant protein. At 15 days after tumor challenge with SP2NS5-21 or SP2/19 (HCV core-expressing) cells, the number of mice that had developed tumors was determined, and the tumor weight was measured. *b* (from *left* to *right*), representative examples of: a mouse immunized with mock DNA and challenged with SP2/NS5-21 cells (A); a mouse immunized with pApNS5 and challenged with SP2/NS5-21 cells (resulting in a tumor-free animal) (B); a mouse immunized with pApNS5 and challenged with SP2/19 (C); and a mouse immunized with recombinant NS5 protein and challenged with SP2/NS5-21 cells (D). Note the large tumor formation on the right flank in mice A, C, and D but not in mouse B.

these nonstructural proteins are far better Ags to stimulate humoral immune responses compared with previous studies by us using the HCV core structural protein (22, 23). Similar to the findings of HCV core, the humoral immune response to the NS3 protein was weak; therefore, it may be necessary to activate APCs by the coadministration of cytokine-expressing plasmids such as IL-2 and granulocyte macrophage CSF to achieve optimal humoral and cellular immune responses, (23, 24). Nevertheless, the generation of inflammatory $CD4^+$ T cell responses with a predominant Th1 phenotype was demonstrated for all three plasmids encoding for NS3, NS4, and NS5. Most important, a specific $CD8^+$ CTL response was generated for NS3 and NS5 with lysis values that have been shown previously to induce protection against a variety of pathogens in animal model systems (18, 19). It was not possible to measure CTL responses to NS4, since we were unable to establish stable NS4-expressing SP2/0 myeloma cell lines. However, CD4 T cell responses and IL-2 and IFN-γ release were in the range observed for NS3 and NS5, and NS4 appears to be an attractive candidate protein for this immunization approach as well. Since no small animal model is currently available for HCV infection, we determined whether the CTL responses generated by DNA-based immunization would protect animals against tumor formation using syngenic SP2/0 tumor cells stably transfected with a cDNA encoding for NS5 protein. Approximately 60% of mice were protected against tumor formation, indicating the in vivo CTL activity produced by this immunization approach. Moreover, tumor weight in those animals that developed tumors was significantly reduced compared with mice immunized with mock DNA or recombinant NS5 protein. This study emphasizes the capability of assessing cellular immune responses against HCV nonstructural proteins in an animal model as measured by inhibition of tumor growth. It should now be possible to determine the fine specificity of CTL epitopes with overlapping peptides using these techniques.

In contrast to the data presented here, DNA immunization using a construct encoding for the HCV core structural protein produced less vigorous cellular and humoral immune responses (22, 23, 25). The envelope region has great sequence diversity among the various genotypes and may not be a good target region because of immunoselection of viral variants known to occur during natural viral infection (22, 25, 26). The NS3 gene encodes for a serine protease that cleaves the viral polyprotein precursor posttranslationally at several junctions and also serves as the viral helicase. The NS5 region encodes for the RNA-dependent RNA polymerase of the virus. Both genomic regions are believed to be highly important and critical for viral replication; therefore, these regions may serve as important molecular targets for antiviral approaches (27–29). Based on both previous clinical studies, which demonstrate the importance of the cellular immune response to the nonstructural proteins with respect to preventing persistent viral infection in humans (6, 7), and the experimental results presented here, which demonstrate that the nonstructural proteins are particularly potent candidates in generating cellular immune responses in mice, we are led to believe that DNA-based immunization with genes encoding for the HCV nonstructural proteins is an attractive approach for the construction of therapeutic and prophylactic vaccines against HCV. However, the clinical efficacy of DNA-based immunization in generating antiviral immune responses against

HCV in humans remains to be established. Finally, it will be important in the future to determine whether different genotypes or subtypes of HCV may circumvent the immune responses induced by one genotype following DNA-based immunization.

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